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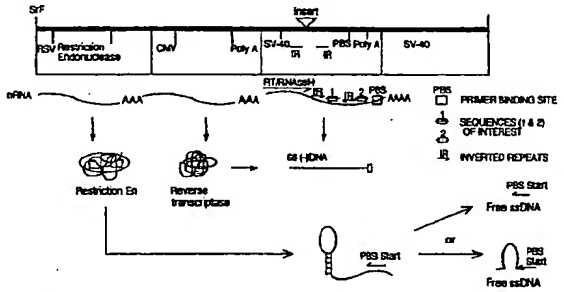
(54) Title: ALTERING GENE EXPRESSION WITH ssDNA PRODUCED *IN VIVO*



(57) Abstract: A methods for altering expression of a target nucleic acid sequence in a target cell by production of single-stranded cDNA (ss-cDNA) in the target cell *in vivo*. The target cell is transfected with a cassette comprising a sequence of interest, an inverted tandem repeat, and a primer binding site 3' to the inverted tandem repeat. Transcription of the cassette by the target cell produces an RNA template which is reverse transcribed to produce ss-cDNA of a specified sequence. A reverse transcriptase/RNAse H coding gene may also be transfected into the target cell. The ss-cDNA is modified to remove all flanking vector sequences by taking advantage of the "stem-loop" structure of the ss-cDNA, which forms as a result of the inverted tandem repeat that allows the ss-cDNA to fold back on itself, forming a double stranded DNA stem. The double-stranded stem contains one or more restriction endonuclease recognition sites and the loop, which remains as ssDNA, is comprised of the sequence of interest, which can be any desired nucleotide sequence. This design allows the double-stranded stem of the stem-loop intermediate to be cleaved by the desired corresponding restriction endonuclease(s) and the loop portion, or sequence of interest, is then released as a linearized, single-stranded piece of DNA. This released (or cleaved) ssDNA piece contains minimal, if any, sequence information either upstream 5' or downstream 3' from the double stranded stem. The resulting ssDNA binds to an endogenous target nucleic acid sequence to alter the expression of that sequence for such therapeutic purposes as gene inactivation using duplex or triplex binding of nucleic acids, site-directed mutagenesis, interruption of cellular function by binding to specific cellular proteins, and interfering with RNA splicing functions.

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ALTERING GENE EXPRESSION WITH ssDNA PRODUCED *IN VIVO*

The present invention relates to alteration of gene expression with a stable DNA construct, conveniently referred to as a cassette, into which a nucleic acid sequence is incorporated for use as a template for subsequent production of that sequence in a prokaryotic or eukaryotic host cell, and a system for expression of that sequence within eukaryotic host cells without (or with minimal) flanking sequences. The construct, or cassette, includes inverted tandem repeats that form the stem of a stem-loop intermediate that functions *in vivo* to cause expression of the sequence, referred to as the sequence of interest, as a single stranded DNA (ssDNA) sequence that binds to or otherwise interacts with a target gene to alter expression of that gene. The expression system of the present invention removes most or all contiguous plasmid (or other vector) sequences from the ssDNA either by stem-loop formation with subsequent termination of a reverse transcription reaction by the stem or by cleavage of the stem-loop intermediate. The ssDNA produced by this method is designed to be complimentary to and/or to otherwise bind to any endogenous nucleic acid sequence target, thereby targeting any desired gene.

Antisense gene therapy has been successfully used in a variety of applications to down regulate gene function. Jain, K.K., Handbook of Gene Therapy, New York: Hofgrefe & Huber Publishing (1998). To date, however, such therapy has been characterized by a number of disadvantages and limitations, some serious, that have decreased the utility of this type of therapy, including the short half-life of the antisense molecule, non-specific effects, uncertainties as to the mode of action of the antisense sequence, and potential toxic effects in animal studies. For instance, antisense oligonucleotides (ODNs) and their analogs must be administered intravenously, which involves problems in cell uptake and distribution (Cossum, P.A., *et al.*, Disposition of the ^{14}C-labeled phosphorothioate oligonucleotide ISIS 2105 after intravenous administration to rats, 267 J. Pharmacol. Exp. Ther. 1181-1190 (1993), Sands, H.,

et al., Biodistribution and metabolism of internally ^{3}H-labeled oligonucleotides. II. 3', 5'-blocked oligonucleotides, 47 Mol. Pharmacol. 636-646 (1995)) as well as toxicity problems due to high blood concentrations (Henry, S.P., *et al.*, Evaluation of the toxicity of ISIS 2302, a phosphorothioate oligonucleotide, in a 4-week study in CD-1 mice, 7 Antisense Nucleic Acid Drug Dev. 473-481 (1997), Henry, S.P., *et al.*, Comparison of the toxicity profiles of ISIS 1082 and ISIS 2105, phosphorothioate oligonucleotides, following subacute intradermal administration in Sprague-Dawley rates, 116 Toxicology 77-88 (1997)).

By far the antisense ODN analogs used most in antisense therapies are phosphorothioates or methylphosphonates. However, phosphorothioate ODNs tend to nonspecifically bind serum and intracellular proteins (Crooke, S.T., *et al.*, Pharmocokinetic properties of several novel oligonucleotide analogs in mice, 227 J. Pharmacol. Exp. Ther. 923-937 (1996), Gao, W.Y., *et al.*, Phosphorothioate oligonucleotides are inhibitors of human DNA polymerases and RNase H: implications for antisense technology, 41 Mol. Pharmacol. 223-229 (1992)), and at higher concentrations, inhibit RNase H activity (Crooke, S.T., *et al.*, Kinetic characteristice of Escherichia coli Rnase H: Cleavage of various antisense oligonucleotide-RNA duplexes, 312 Biochem. J. 599-608 (1995)). Phosphorothioate ODNs have a lower Tm (an average of 0.5°C per base pair) for RNA than does DNA (Crooke, S.T. and B. LeBleu, Antisense research and application, Boca Raton: CRC Press (1993)). This lower Tm requires that phosphorothioate ODNs typically be longer than phosphodiester DNA oligonucleotides for effective binding. However, an increase in the length of the ODN can cause a loss of hybridization specificity (Toulme, J.J., *et al.*, Antisense technology: A practical approach, in C. Lichtenstein and W. Nellen (Eds.), New York: IRL Press, pp. 39-74 (1997)). In addition, methylphosphonate ODNs do not activate RNase H enzyme activity (Maher, L.J, *et al.*, Inhibition of DNA binding proteins by oligonucleotide-directed triple helix formation, 245 Science 725-730 (1989), Miller, P.S., Oligodeoxynucleotides: Antisense inhibitors of gene expression, in J.S. Cohen (Ed.), Boca Raton: CRC Press, p. 79 (1989)) and are eliminated rapidly (Chen, T.L., *et al.*, Disposition and metabolism of oligodeoxynucleoside

methylphosphonate following a single i.v. injection in mice., 18 Drug Metab. Dispos. 815-818 (1990)).

Another approach to gene therapy is to administer molecules that have catalytic activity against the gene and/or the transcriptional product of the gene. Ribozymes comprise only RNA molecules, which can catalyze the cleavage of specific mRNA sequences, and are thought to be potentially more efficient than antisense ODNs because of their catalytic capability (Woolf, T.M., To cleave or not to cleave: Ribozymes and antisense, 5 Antisense Res. Dev. 227-232 (1995)). Ribozymes have been used as inhibitors of gene expression and viral replication (Jain, *supra* (1998)). Unlike antisense ODNs, ribozymes can be delivered either endogenously, such as by using viral vectors, or exogenously. However, ribozymes have limited stability due to degradation by RNases *in vivo* (Jain, *supra* (1998)).

Using *in vitro* selections, several small single-stranded DNAs have recently been demonstrated to catalyze the cleavage of RNA (Breaker, R.R., Catalytic DNA: In training and seeking employment, 17 Nature Biotechnology 422-423 (1999)), thereby offering the promise of targeted activity against specific genes. The patent and scientific literature describes a number of these short deoxynucleic acid sequences that have been shown to have catalytic activity (*see*, Breaker, R.R. and G.F. Joyce, 1 Chem. Biol. 223-229 (1994); Cuenoud, B. and J.W. Szostak, 375 Nature 611-613 (1995); Santoro, S.W. and G.F. Joyce, 94 Proc. Natl. Acad. Sci. USA 4262-4266 (1997); Faulhammer and M. Famulok, 269 J. Molec. Bio. 188-203 (1997); Carmi, N, *et al.*, 95 Proc. Natl. Acad. Sci. USA (1998); Li, Y. and R.R. Breaker, 96 Proc. Natl. Acad. Sci. USA 2746-2751 (1999) and U.S. Patent Nos. 5,807,718 and 5,910,408), including the so-called "10-23 DNA enzyme" and other ssDNA sequences that act, for instance, as copper-dependent DNA ligases and calcium-dependent DNA kinases. The catalytic efficiency of such sequences has been demonstrated for cleaving mRNA targets at 10^9 m^{-1}/min^{-1} in the presence of divalent magnesium, thereby offering the opportunity for targeted destruction of substrate molecules (*see, for instance*, R.R. Breaker, *supra* (1999)). Although the art appears to recognize the potential for use of this enzymatic activity for therapeutic purposes, so far as is known, no system is

available for producing these target-specific enzymatic nucleic acid sequences to produce a therapeutic effect *in vivo*.

There are, therefore, no systems available that utilize the potential advantage of the efficient catalytic activity of these enzymatic nucleic acid sequences for altering gene expression. It is, therefore, an object of the present invention to provide a DNA construct that directs the synthesis of ssDNA containing a sequence that specifically cleaves mRNA targets *in vivo* to alter the expression of the gene producing that target mRNA.

Because secondary structure folding may be critical to the catalytic function of the enzymatic sequence of the ssDNA, it is another object of the present invention to provide methods, and DNA constructs, for producing ssDNA including such a DNA enzyme sequence of any desired nucleotide sequence within eukaryotic cells without undesirable intervening or flanking nucleotide bases so as to preserve the enzymatic function of the ssDNA against a target nucleic acid, for use in altering expression of a gene including that target nucleic acid.

Another object of the present invention is to provide a method, and a DNA construct utilized in such methods, for production of ssDNA within eukaryotic cells that contains DNA enzyme sequences for overcoming the significant problems encountered by the use of standard oligonucleotide delivery methods for therapeutic purposes.

Another object of the present invention is to provide a method, and a DNA construct, for producing ssDNA of any nucleotide sequence *in vivo* that functions as (but is not limited to) an inhibitory nucleic acid for, for instance, binding to mRNA in an anti-sense fashion to down regulate a gene product or a viral gene product of interest or binding to and inhibiting a specific cellular function, for instance, by binding to proteins that recognize a nucleic acid sequence.

Another object of the present invention is to provide a method, and a DNA construct, for producing ssDNA designed to favor binding to duplex (native DNA) to form triplex structures that may interfere with normal gene transcription and regulation.

Another object of the present invention is to produce ssDNA within eukaryotic cells for the purpose of disrupting one or more cell functions.

Yet another object of the present invention is to provide a method, and a DNA construct, for producing ssDNA into which secondary structures are designed so that the ssDNA oligonucleotides bind to and/or otherwise inhibit or activate various cellular functions that rely on the catalytic action of a protein or on nucleic acid protein interaction such as transcription, translation, and DNA replication.

Another object of the present invention is to provide a method, and a DNA construct, for producing ssDNA *in vivo* for site-directed mutagenesis or gene knockout for therapeutic applications.

Another object of the present invention is to provide a method, and a DNA construct, for producing ssDNA of precisely defined nucleotide composition that favors site-specific insertion into a genome for therapeutic purposes.

Yet another object of the present invention is to provide a method, and a DNA construct, for producing ssDNA that is complimentary to any endogenous nucleic acid target for use in altering expression of a gene including the nucleic acid sequence target.

Another object of the present invention is to provide a method, and DNA expression utilized in such a method, for *in vivo* production of ssDNA including a sequence exhibiting catalytic activity against mRNA targets for transfection into eukaryotic cells that overcomes the obstacles to delivery of direct administration of ssDNA by lipofection, direct cellular uptake, and/or microinjection.

Another object of the present invention is to provide all enzymatic functions that are necessary to produce ssDNA *in vivo* containing a sequence with enzymatic activity against a target mRNA of choice within a single or dual plasmid expression system.

Another object of the present invention is to provide a method, and pharmacologically acceptable compositions, for delivering an inhibitory nucleic acid sequence including a sequence with enzymatic activity to target cells in a manner which produces a therapeutic effect.

This listing of the objects of the present invention is not intended to be a list of all the objects of this invention. There are a vast number of other cellular functions that are mediated by the cellular genome which, in the interest of brevity and practicality, are not mentioned here and which are amenable to regulation by *in vivo* production of ssDNA. For instance, exonucleases digest ssDNA much more aggressively than double-stranded DNA (dsDNA). Consequently, another object of the present invention is to provide a ssDNA construct, and a method of producing that construct *in vivo*, that is not as susceptible to degradation by native exonucleases in the cell as dsDNA. It can be seen from this illustration that this list of some of the objects of the present invention is provided for exemplification and is not intended to limit the scope of the invention.

These objects are provided by a method of altering expression of an endogenous nucleic acid target sequence in a target cell comprising the steps of introducing a cassette comprised of a sequence of interest flanked by an inverted tandem repeat and a 3' primer binding site (PBS) into a target cell and reverse transcribing the mRNA transcript of the cassette from the PBS to release a single-stranded cDNA transcript in the cell. The sequence of interest is comprised of a nucleic acid sequence that produces a sequence of nucleic acids that binds to an endogenous target nucleic acid sequence when reverse transcribed to alter expression of the target sequence.

Several embodiments of the invention are illustrated in the figures, in which Figure 1 is a schematic illustration of a production of ss-cDNA in a host cell in accordance with the present invention.

Figure 2 is a schematic illustration of the stem-loop intermediate formed by the method illustrated in Fig. 1.

Figure 3 is a schematic illustration of the pssXA plasmid comprising a first component of a first embodiment of the expression system of the present invention. To make pssXA, reverse transcriptase (RT) and *Mbo*II genes were subcloned into the mammalian expression vector pBK-RSV (Stratagene) and expressed as a single polypeptide. The RT and *Mbo*II domains are separated by a histidine-rich linker.

Figures 4A and 4B are schematic illustrations of the pssXB plasmid comprising a second component of the first embodiment of the expression system of the present invention (Fig. 4A) that includes the sequence of interest and contains (1) the MoMuLV reverse transcriptase pomoter region, (2) two *Not*I sites, one *Pac*I and one *BamH*I, for the subcloning of the DNA sequence of interest, and (3) the tandem inverted repeats, IR-L and IR-R, and the sequence of the insert region of the pssXB plasmid (Fig. 4B).

Figure 5A is a schematic illustration of the pssXC plasmid comprising a second embodiment of the expression system of the present invention and including the 10-23 DNA enzyme sequence illustrated schematically in Fig. 5B.

Figures 6A and 6B represent schematic illustrations of the pssXD plasmid comprising a third embodiment of the expression system of the present invention Fig. 6A) and an elarged portion of the pssXD plasmid (Fig. 6B).

Figure 7 shows the result of a PCR assay for RT activity in a pssXA transfected cell lysate. Lanes 1 and 2: A549 cells transiently transfected with the pBK-RSV vector; lanes 3 and 4: A549 cells transiently infected with pssXA; lanes 5 and 6: A549 cells stably transfected with pssXA (E10). Before PCR amplification, reverse transcription reaction was carried out for 10 (lane 1, 3, and 5) or 30 minutes (lane 2, 4, and 6), repectively, at 37°C.

Figure 8 shows the result of an assay for detection of ssDNA by PCR analysis. Total RNA isolated from either E10 cells, transiently transfected with pssXB vector, pssXB-I or pssXB-II. Before PCR amplification, total RNA was pre-treated with either S1 nuclease (lanes 1 and 3) or RNase (lanes 2, 4, and 5) for 30 minutes at 37°C. lanes 1 and 2: pssXB-I; lanes 3 and 4: pssXB-II; lane 5: pssXB vector.

Figure 9 shows the results of a dot blot analysis for detection of ssDNA. 1: E10 cells transfected with pssXB-I; 2. E10 cells transfected with pssXB-II; 3: E10 cells; 4: A549 cells.

Figure 10 shows a bar graph quantitating a Northern blot of a ssDNA-producing vector constructed in accordance with the present invention producing an antisense sequence against c-raf kinase. Lanes 1-3: cells harvested 24 hrs after

transfection; lanes 4-6: cells harvested 48 hrs after transfection. Lane 1: E10 cells transfected with pssXB vector; lanes 2 and 5: E10 cells transfected with pssXB-I; lanes 3 and 6: E10 cells transfected with pssXB-II.

Figure 11 shows the results of a dot blot analysis for detection of ssDNA in A549 cells transfected with control pssXD-I or pssXD-II containing the *c-raf* DNA enzyme sequence. No detectable signal was produced in the presence of S1 nuclease due to the specific degradation of ssDNA enzyme by S1 nuclease.

Figure 12 shows the results of quantitative RT-PCR to determine whether ssDNA expressed in A549 cells transfected with pssXD-II altered *c-raf* mRNA levels. Lane 1: control pssXD-I; Lane 2: pssXD-II.

Figure 13 shows the results of a Western blot for suppression of *c-raf* protein expression in A549 cells transfected with pssXD-I or pssXD-II. Lane 1: pssXD-II; Lane 2: control pssXD-I; Lane 3: untransfected cells.

Figure 14 shows the results of a Western blot for genomic DNA cleavage for induction of cell apoptosis by suppression of *c-raf* gene expression. Lane 1: pssXD-II; Lane 2: control pssXD-I; Lane 3: untransfected cells.

Figure 15 shows the results of a Western blot for PARP cleavage for induction of cell apoptosis by suppression of *c-raf* gene expression. Lane 1: pssXD-II; Lane 2: control pssXD-I; Lane 3: untransfected cells.

In this description of the present invention, methods and nucleic acid constructs are described for producing single-stranded deoxyribonucleic acid (ss-cDNA) oligonucleotides of virtually any predefined or desired nucleotide base composition *in vivo* in yeast, prokaryotic cells, and/or eukaryotic cells, with or without flanking nucleotide sequences, for use in altering the expression of a target gene. Methods and constructs are described that use biological rather than the *in vitro*, or artificial, synthesis of ss-cDNA of desired nucleotide base composition. Because biological, i.e., enzymatic reactions, are used in these methods, they are applicable to any *in vivo* system.

In one embodiment, the expression system of the present invention comprises a vector (as used herein, the term "vector" refers to a plasmid or modified viral or non-

viral recombinant biological construct used to deliver and manipulate synthesized and/or naturally occurring nucleic acid sequences) designed to produce any sequence of interest as a ss-cDNA molecule, preferably free of most contiguous vector sequences, within mammalian cells. The vector system contains all the necessary enzymatic functions and signaling instructions for producing ss-cDNA in the host cell. The host cell to which the vector of the present invention is delivered produces an RNA transcript (Fig. 1), driven by an eukaryotic promoter, that is used as a template to direct the synthesis of any desired single-stranded DNA sequence (a "sequence of interest").

In more detail, a first expression system in which the vector comprises two plasmids that are co-transfected into a suitable host cell, which can be yeast or any prokaryotic or eukaryotic cell, to produce the ssDNA sequence of interest in the cell for altering gene expression is described herein. A second expression system is also described that comprises a single plasmid including the sequence of interest that is transfected into a suitable host cell for production of the ssDNA sequence of interest in the cell for altering gene expression.

The ssDNA produced *in vivo* using the expression systems described herein may be an inhibitory nucleic acid. Inhibitory nucleic acids may be ssDNA synthesized from the mRNA template or the mRNA template itself, which can specifically bind to a complementary nucleic acid sequence. By binding to the appropriate target nucleic acid sequence, an RNA--RNA, a DNA--DNA, or RNA--DNA duplex or triplex is formed. More commonly, these nucleic acids are often termed "antisense" because they are usually complementary to the sense or coding strand of the gene, but the "sense" sequence is also utilized in the cell for therapeutic purposes. The term "inhibitory nucleic acids" as used herein, therefore, refers to both "sense" and "antisense" nucleic acids.

By binding to a target nucleic acid, an inhibitory nucleic acid alters the function of the target nucleic acid. This alteration (usually an inhibitory effect) results from, for example, blocking DNA transcription, processing or poly(A) addition to mRNA, DNA replication, translation, or promoting inhibitory mechanisms of the cells (such as

promoting RNA degradation). Inhibitory nucleic acid methods therefore encompass a number of different approaches to altering gene expression. These different types of inhibitory nucleic acid technologies are described in Helene, C. and J. Toulme, 1049 Biochim. Biophys. Acta. 99-125 (1990), hereinafter referred to as "Helene and Toulme," and which is incorporated herein in its entirety by this specific reference thereto.

In brief, inhibitory nucleic acid therapy approaches can be classified into (1) those that target DNA sequences, (2) those that target RNA sequences (including pre-mRNA and mRNA), (3) those that target proteins (sense strand approaches), and (4) those that cause cleavage or chemical modification of the target nucleic acids such as the ssDNA enzymes, including the so-called "10-23 enzyme" as described herein. The first approach contemplates several categories. Nucleic acids are designed to bind to the major groove of the duplex DNA to form a triple helical or "triplex" structure. Alternatively, inhibitory nucleic acids are designed to bind to regions of single stranded DNA resulting from the opening of the duplex DNA during replication or transcription. More commonly, inhibitory nucleic acids are designed to bind to mRNA or mRNA precursors. Inhibitory nucleic acids are also used to prevent maturation of pre-mRNA. Inhibitory nucleic acids may be designed to interfere with RNA processing, splicing or translation. In the second approach, the inhibitory nucleic acids are targeted to mRNA. In this approach, the inhibitory nucleic acids are designed to specifically block translation of the encoded protein. Using this second approach, the inhibitory nucleic acid is used to selectively suppress certain cellular functions by inhibition of translation of mRNA encoding critical proteins. An example of such an inhibitory nucleic acid is the sequence that is complementary to regions of c-myc mRNA, which inhibits c-myc protein expression in a human promyelocytic leukemia cell line, HL60, which overexpresses the c-myc proto-oncogene (Wickstrom E. L., *et al.*, 85 Proc. Natl. Acad. Sci. USA 1028-1032 (1988) and Harel-Bellan, A., *et al.*, 168 Exp. Med. 2309-2318 (1988)). As described in Helene and Toulme, inhibitory nucleic acids targeting mRNA have been shown to work by several different mechanisms to inhibit translation of the encoded protein(s).

Inhibitory nucleic acids can also utilize the third approach of designing the "sense" strand of the gene or mRNA to trap or compete for enzymes or binding proteins involved in mRNA translation as described in Helene and Toulme. Lastly, inhibitory nucleic acids are used to induce chemical inactivation or cleavage of the target genes or mRNA. Chemical inactivation occurs, for instance, by induction of crosslinks between the inhibitory nucleic acid and the target nucleic acid within the cell and by the method contemplated herein, namely, the cleavage of the target nucleic acid by the sequence having enzymatic activity that is incorporated into the cassette of the present invention.

In brief, in a first aspect, the present invention comprises a set of genetic elements adapted for delivery into a cell to produce ssDNA *in vitro* or *in vivo* for altering gene expression, an expression system comprising the set of genetic elements, and one or more stably transfected cell(s) comprising the set of genetic elements. The set of genetic elements is incorporated into an expression system for delivery into the cell and includes

(A) an RNA dependent DNA polymerase (reverse transcriptase) gene, and

(B) a cassette including (1) an inverted tandem repeat (IR), (2) one or more sequences of interest located (a) between the inverted repeat (IR), (b) 3' to the IR, or (c) both between the IR and 3' to the IR and (3) a primer binding site (PBS) for the reverse transcriptase that is located 3' to the IR as shown in Fig. 2.

Although not required, the expression system also preferably includes the functions and signaling instructions for transcription of these components *in vivo* and the functions and signaling instructions for translation of the reverse transcriptase (RT) gene. Additional elements that are optionally included in the set of genetic elements of the present invention may include one or more of an RNAse gene, usually associated with the RT gene, a restriction endonuclease (RE) gene (for a purpose described below), a downstream polyadenylation signal sequence for expression in eukaryotic cells so that the mRNA produced by the sequence of interest includes a poly(A) tail (see Fig. 1),

and a DNA sequence having enzymatic activity when the linearized ssDNA folds into the appropriate secondary configuration. Although the present invention is not so limited, in one embodiment of the set of genetic elements, the DNA enzymatic sequence is located within a sequence of interest, regardless of whether the sequence of interest is located between the inverted repeat (IR) or between the 3' aspect of the IR and the PBS.

In a first embodiment of the expression system described herein, a vector system is provided that comprises a two plasmids, and the above-described set of genetic elements that is adapted for delivery to the cell to produce ssDNA *in vivo* includes the RNA-dependent DNA polymerase (reverse transcriptase) gene, which additionally contains an RNAse H gene, linked with a histidine-proline linker to a restriction endonuclease gene. These genes were constructed and inserted into a plasmid vector that contains the necessary transcriptional and translational control elements along with polyadenylation tailing sequences. This plasmid is referred to herein as the "A" plasmid, pssXA, as shown in Fig. 3. A second, "B" plasmid was constructed which, in the embodiment described herein, includes the three above-listed elements of the cassette, namely, a primer binding sequence (PBS) matched to the reverse transcriptase (RT), a sequence of interest (SOI), and an inverted repeat (IR). In this second plasmid, exemplified by the plasmid pssXB shown in Fig. 4, the SOI is located either between the inverted tandem repeats or in a 5' position (with respect to the mRNA transcript) to the PBS, the PBS being located at the most 3' aspect of the mRNA transcript. In other words, the SOI is located (1) between the IR, (2) between the IR and the PBS, and/or (3) both between the IR and between the IR and the PBS, and as will be described below, two B plasmids are described herein, one (pssXB-1) with the SOI between the IR (e.g., *Not*I sites) and the other (pssXB-II) with the SOI between the IR and the PBS (e.g., cloned into the *Pac*I/*Bam*HI sites). Like plasmid A, plasmid B also includes a combination of transcriptional control elements. However, in another preferred embodiment herein, the B plasmid does not include (or require) translational control elements since no protein product is produced from this construct.

In another embodiment described herein, the expression system of the present invention comprises a single plasmid vector, shown schematically in Figs. 5 and 6 and designated as plasmids pssXC and pssXD, respectively, in which the above-described set of genetic elements is incorporated. The components of the B plasmid described above, e.g., the PBS, SOI, and IR, reside in the untranslated 3' portion of the RT polyprotein in this C plasmid. In other words, when the RT-RNAse H component of the C plasmid is transcribed under control of an appropriate promoter (in the embodiments described herein, the RSV promoter was utilized), the resulting mRNA transcript contains the coding region for the RT-RNAse H polyprotein and, at the end of translation at the stop signals, the additional mRNA transcript contains (3' to this translated protein) the elements from the B plasmid with further 3' downstream signaling events for polyadenylation signals, which remain intact from the RT-RNAse H component.

The particular single plasmid expression system described herein does not contain the restriction endonuclease (RE) gene, and therefore does not digest the stem of the stem-loop intermediate formed by the inverted repeats. Consequently, the SOI (including the DNA enzyme) is inserted into either the C or D plasmids only in a 3' position to the IR and unwanted vector sequences are removed by premature truncation of the ss-cDNA product as the transcript encounters the relatively stable stem of the stem-loop intermediate and is unable to continue transcribing ss-cDNA from the mRNA transcript. More specifically, as will be made apparent in the following description, each SOI was inserted only within the *PacI*/*BamHI* restriction sites of the pssXC and pssXD plasmids.

As will also be apparent from the following description of the B, C, and D plasmids, the plasmids include cloning sites for insertion of the SOI. Both *NotI* sites (located between the IR) and *PacI*/*BamHI* (3' to the IR, e.g., between the IR and the PBS) sites are provided in the preferred embodiment of the B plasmid described herein. The C and D plasmids described herein include only the *PacI*/*BamHI* sites for this purpose. However, those skilled in the art who have the benefit of this disclosure will recognize that these particular cloning sites were chosen for the particular systems

described herein and that other cloning sites may be equally useful for this same purpose. The A plasmid comprising the two plasmid vector system described herein was not intended to include the SOI, but those skilled in the art will also recognize that, if a two plasmid vector system is to be used, the elements of the set of genetic elements of the present invention, and particularly the SOI, may be inserted into either plasmid as may be convenient.

The nucleic acid sequence that is referred to herein as a cassette provides the template for synthesis of ss-cDNA in target cells. It is this element that includes the SOI, IR, and PBS. As is the case for the other elements of the set of genetic elements of the present invention, this genetic element is preferably regulated by an appropriate wide spectrum or tissue-specific promoter/enhancer, such as the CMV promoter, or combination of promoters/enhancers, located upstream of the genetic element. Also as is the case for the other genetic elements, the promoter/enhancer can either be constitutive or inducible promoter. Those skilled in the art who have the benefit of this disclosure will recognize that a number of other eukaryotic promoters may be used to advantage to control expression of the SOI including SV-40, RSV (non-cell type specific) or tissue specific glial fibulary acidic protein (GFAP).

The primer binding site (PBS) for initiation of priming for cDNA synthesis is located between the 3' IR and the polyadenylation signal. The PBS is a sequence that is complementary to a transfer RNA (tRNA) which is resident within the eukaryotic target cell. In the case of the mouse Maloney reverse transcriptase (MoMULV RT) described herein as being utilized in conjunction with the present invention, the PBS takes advantage of the proline tRNA. The PBS utilized in connection with the presently preferred embodiment of the invention that is described herein was taken from the actual 18 nucleotide sequence region of mouse Moloney virus. Shinnick, T.M., *et al.*, Nucleotide sequence of Moloney murine leukemia virus, 293 Nature 543-548 (1981). In the case of the RT gene from human immunodeficiency virus that was also tested as noted below, the PBS used was taken from the nucleotide sequence of HIV. Y. Li, *et al.*, 66 J. Virology 6587-6600 (1992). In short, any PBS that is matched to a particular RT is utilized for this purpose. The PBS is exclusively

recognized by a primer tRNA that is endogenous to the target cells. Each tRNA has the ability to recognize a unique sequence (i.e., codon) on the mRNA transcript coding for an amino acid, and has the ability to covalently link to a specific amino acid (i.e., the tRNA becomes "charged" when bound to a specific amino acid). However, a primer tRNA, when bound to the mRNA transcript PBS and not covalently linked with an amino acid (i.e., "uncharged"), may be used to initiate ssDNA synthesis by the RT. For example, the MoMULV RT used in the examples described herein recognizes and uses an uncharged lysine tRNA that in turn recognizes and binds to its unique sequence in the PBS. Thus, each PBS incorporated into the expression system of the present invention must contain the unique sequence recognized by the primer tRNA, and the primer tRNA must be a primer tRNA that is recognized by the particular RT utilized.

Other retroviral RT/RNAse H genes may be used to advantage in connection with the present invention, it being preferred that the RT/RNase H gene be an RT/RNase H gene that is regulated by an appropriate upstream eukaryotic promoter/enhancer such as the CMV or RSV promoter for expression in human cells. RNA-dependent DNA polymerase/RT genes suitable for use in connection with the present invention include those from retroviruses, strains of hepatitis B, hepatitis C, bacterial retron elements, and retrons isolated from various yeast and bacterial species. As found in nature, these RNA-dependent DNA polymerases usually have an associated RNase H component enzyme within the same coding transcript. However, the present invention does not require the naturally-occurring RNase H gene for a particular RT. In other words, those skilled in the art will recognize from this disclosure that various combinations of RT and RNase H genes can be spliced together for use in connection with the present invention to fulfill this function and that modifications and/or hybrid versions of these two enzyme systems are available and/or known to those skilled in the art which will function in the intended manner. Those skilled in the art will also recognize that the target cell may itself have sufficient endogenous RNase H to fulfill this function. Similarly, those skilled in the art will recognize that the target cell may itself have sufficient endogenous RT activity from, for instance, prior retroviral infection, to fulfill this function.

The RT/RNase H gene also preferably includes a downstream polyadenylation signal sequence so that the mRNA produced from the RT/RNase H gene includes a 3' poly(A) tail for mRNA stability. As known to those skilled in the art, multiple poly(A) tails are available and are routinely used for production of expressed eukaryotic genes.

Those skilled in the art will also recognize that a number of tissue-specific or wide spectrum promoters/enhancers, or combinations of promoters/enhancers other than those listed above may also be used to advantage to regulate the RT/RNAse H gene, the RE gene (if utilized), and the sequence of interest. Although a list of all available promoters/enhancers is not needed to exemplify the invention, as noted above, the promoters/enhancers may be constitutive or inducible and may include the CMV or RSV (non-cell type specific) or GFAP (tissue specific) promoters/enhancers listed here and many other viral or mammalian promoters. Representative promoters/enhancers that are appropriate for use in connection with the cassette of the present invention may include, but are not limited to, HSVtk (S.L. McKnight, *et al.*, 217 Science 316 (1982)), human ß-globulin promoter (R. Breathnach, *et al.*, 50 Ann. Rev. of Biochem. 349 (1981)), ß-actin (T. Kawamoto, *et al.*, 8 Mol. Cell Biol. 267 (1988)), rat growth hormone (P.R. Larsen, *et al.*, 83 Proc. Natl. Acad. Sci. U.S.A. 8283 (1986)), MMTV (A.L. Huang, *et al.*, 27 Cell 245 (1981)), adenovirus 5 E2 (M.J. Imperiale, *et al.*, 4 Mol. Cell. Biol. 875 (1984)), SV40 (P. Angel, *et al.*, 49 Cell 729 (1987)), α-2-macroglobulin (D. Kunz, *et al.*, 17 Nucl. Acids Res. 1121 (1989)), MHC class I gene H-2kb (M.A. Blanar, *et al.*, 8 EMBO J. 1139 (1989)), and thyroid stimulating hormone (V.K. Chatterjee, *et al.*, 86 Proc. Natl. Acad. Sci. U.S.A. 9114 (1989)).

The RT produced in the cell synthesizes a complementary DNA (cDNA) using as the template the genetic element including the SOI described below. The RNase H activity of the RT degrades the mRNA template component of the RNA/cDNA hybrid to produce a ss-cDNA *in vivo*.

The gene encoding the RE (used in the two plasmid expression system and not a required component of that system) may be any of several genes which encode for REs, and preferably those that are controlled by one or more constitutive or inducible

wide spectrum and/or tissue-specific promoters/enhancers such as those listed above. The particular REs tested were *Mbo*II and *Fok*I, but those skilled in the art who have the benefit of this disclosure will recognize that any RE (type I, II, IIS, or III) site may be included in the IR. These enzymes "clip," or digest, the stem of the stem-loop intermediate described below to linearize the SOI as single-stranded DNA.

Although expression of the RE gene may be regulated by an appropriate constitutive or inducible promoter/enhancer located upstream from the restriction endonuclease gene such as the CMV or RSV promoter for expression in human cells, in plasmid pssXA, the RE gene (*Mbo*II) is linked to the RT-RNAse H polypeptide. The RE gene also preferably includes a downstream polyadenylation signal sequence so that the mRNA transcript from the RE gene will have a 3' poly(A) tail.

The cassette of the present invention also comprises an inverted tandem repeat (IR). After digestion of the mRNA from the mRNA-cDNA heteroduplex by RNAse H and the release of the ss-cDNA, the IR causes the ss-cDNA to fold back upon itself to form the stem of a stem-loop structure, the stem structure being comprised of double stranded, anti-parallel DNA, in the manner described in U.S. Patent No. 6,054,299 and as shown in Fig. 2, after the cassette is transcribed in the cell and after the RT/RNase H produced by transcription of the genes produces the ss-cDNA sequence of interest from the mRNA transcript in the cell. One or more RE site(s) which is cut by the RE produced from the RE gene (in the case of those plasmids that include an RE gene) may be designed into the double stranded portion, i.e., the IR, that forms the stem of the stem-loop intermediate. The ss-cDNA which is produced is transcribed with the encoded 5' and 3' regions flanking the stem (made up of the IR) and a loop containing the SOI. The stem is then cut (also termed digested or cleaved) by any of the many RE enzymes that recognize the cut site designed into the stem (note that the endonuclease recognition site may be designed into the stem even though the RE gene is not included in the vector system of the present invention) to release the ss-cDNA loop (see Fig. 1). The loop portion of the ss-cDNA, which does not form any apparent duplex DNA, is immune to RE activity since REs recognize only double stranded DNA as a target substrate.

As noted above, those skilled in the art will recognize that the RE site(s) need not be designed into the IR which forms the stem of the stem-loop intermediate if it is desired to produce ssDNA from an SOI located between the PBS and the IR, with transcription of the cassette terminating at the stem formed by the IR. Another option is to design the IR to contain eukaryotic, prokaryotic, or viral protein DNA binding sites, which can act to competitively titer out selected cellular proteins. Combinations of restriction sites or other sequence specific elements may be included in the IR depending on the base pair composition chosen for the IR such that linear or precisely cut stem-loop intermediate forms of ssDNA are produced. It is generally preferred to use synthetically constructed sequence specific elements in the IR since it is unlikely that a naturally occurring inverted repeat would have the properly aligned restriction sites.

As noted above, the cassette which comprises one of the elements of the set of genetic elements of the present invention may also include a DNA sequence with catalytic activity. Because of the inclusion of the so-called "DNA enzyme" in the cassette (and in the embodiment described herein, the DNA enzyme is located within the sequence of interest), the present invention is used to particular advantage when the sequence of interest serves as the template for synthesis of an inhibitory nucleic acid that is an antisense sequence. For that reason, the examples set out herein describe production of an antisense SOI as set out in Fig. 5B including a sequence having enzymatic activity against mRNA including a *c-raf* cleaving enzyme designed specifically to bind to the 3' untranslated region of the *c-raf* mRNA, which is targeted by antisense ISIS 5132 (Monia, B.P., *et al.*, 2 Nature Medicine 668-675 (1996), hereby incorporated into the present specification in its entirety by this specific reference). The two 9 bp target specific binding arms were flanked by the 15 bp catalytic domain (Santoro, S.W. and G.F. Joyce, Mechanism and utility of an RNA-cleaving DNA enzyme, 37 Biochemistry 13330-13342 (1998), also incorporated into the present specification in its entirety by this specific reference). Compatible restriction sites were added to the DNA enzyme oligonucleotides so that they could be

inserted into either *Not*I sites or *Pac*I and *BamH*I, and the resulting plasmids were designated as pssXB-I and pss-XB-II, respectively.

Those skilled in the art will recognize that the present invention is not limited just to antisense sequences, that the antisense sequence need not necessarily contain a nucleic acid sequence having catalytic activity, and that the inhibitory nucleic acid sequence could also be any of the other types of inhibitory nucleic acid sequences described above. The above-described SOI was chosen for demonstration of the present invention because the *c-raf* kinase in A549 lung carcinoma cells system has been well characterized (Monia, *et al.*, *supra* (1996)). Raf protein is a serine/threonin protein kinase shown to act as a direct downstream effector of ras protein within the MAP kinase signaling pathway with downstream activiation of MEK1/MEK2 and subsequent activiation of ERK1 and ERK2 (Daum, G., *et al.*, The ins and outs of raf kinases, 19 Trends Biol. Sci. 474-480 (1994)). A number of solid tumors and leukemias have been demonstrated to harbor either mutations in ras or have upregulations in MAP kinase signal pathways. Signal transduction pathways such as *c-raf* related tumors have been attractive targets for oncological therapies and the phosphorothioate ODN ISIS 5132, noted above, has been demonstrated to be a potent antisense inhibitor (Monia, *et al.*, *supra* (1996)). Further, ISIS 5132 has been shown to induce apotosis (Lau, Q.C., *et al.*, 16 Oncogene 1899-1902 (1998), also incorporated into the present specification in its entirety by this specific reference) and appears to represent a potential effective treatment against such tumors. This antisense ODN has recently entered Phase I clinical trials (O'Dwyer, P.J., *et al.*, C-raf-1 depletion and tumor responses in patients treated with the c-raf-1 antisense oligonucleotide ISIS 5132 (CGP 69846A), 5 Clinical Cancer Res. 3977-3982 (1999)), and may prove to be useful in treating *c-raf*-related tumors. Other SOIs that have been cloned into plasmids for expression using the expression system of the present invention include the partial sequence of the 23rd codon of h-ras antisense binding sequence with the 10-23 DNA enzyme sequence (Santoro and Joyce, *supra* (1997)) inserted between the 5' and 3' complimentary sequences, the partial sequence of pleiotropin antisense binding sequence with the 10-23 DNA enzyme sequence inserted

between the 5' and 3' complimentary sequences, and the partial sequence of tat antisense binding region of the SIV sequence with the 10-23 DNA enzyme sequence inserted between the 5' and 3' complimentary sequences. Although each of these sequences included the DNA enzyme sequence, those skilled in the art will recognize from this disclosure that the DNA enzyme sequence need not be included with these, or any other, SOIs.

The nucleic acid sequence having enzymatic activity utilized in the method of altering gene expression described herein is the 10-23 DNA enzyme (Santoro and Joyce, *supra* (1997)). The enzymatic sequence is inserted into the cassette in either or both of the two locations, e.g., (a) between the IR and inside the SOI (at the *Not*I site) or (b) inside the second SOI that is located 3' to the IR and 5' to the PBS (at the *Pac*I/*BamH*I sites). Either way, the resulting aptamer is specific for the target of the SOI and is therefore used to target other DNA sequences, mRNA sequences, and any other suitable substrate, to inhibit or change DNA or mRNA splicing mechanisms, or even to directly alter the cellular genome in a specific manner.

Those skilled in the art will recognize from this disclosure that any DNA sequence having enzymatic activity will function for the intended purpose when inserted into the cassette of the present invention. A number of nucleic acid sequences with enzymatic activity have been reported in the literature, including:

sequences having RNAse activity such as the so-called "10-23" and "8-17 enzymes" (Santoro, S.W. and G.F. Joyce, *supra* (1997)) and other metal-dependent RNAses (Breaker, R.R. and G.F. Joyce, 1 Biol. Chem. 223-229 (1994) and Breaker, R.R. and G.F. Joyce, 2 Biol. Chem. 655-660 (1995)) and histidine-dependent RNAse (Roth, A. and R.R. Breaker, 95 Proc. Natl Acad. Sci. USA 6027-6031 (1998));

sequences having DNAse activity such as copper-dependent DNAse (Carmi, N., *et* al., 3 Chem. Biol. 1039-1046 (1996), Carmi, *et al.*, *supra* (1997); Sen, D. and C.R. Geyer, 2 Curr. Opin. Chem. Biol. 680-687 (1998)) and the DNAses which required divalent metal ions as cofactors or hydrolyzed

the substrate independently of divalent metal ions reported in Faulhammer, D. and M. Famulok (269 J. Molec. Bio. 18-203 (1997));

sequences with DNA ligase activity such as copper-dependent DNAse (Breaker, R.R., 97 Chem. Rev. 371-390 (1997)) and zinc-dependent E47 ligase (Cuenoud, B. and J.W. Szostak, 375 Nature 611-613 (1995));

sequences with DNA kinase acitivity such as calcium-dependent DNA kinase (Li, Y. and R.R. Breaker, 96 Proc. Natl. Acad. Sci. USA 2746-2751 (1999)); and

sequences with RNA kinase acitivity such as calcium-dependent DNA kinase (Li, Y., *supra* (1999)).

Generally, it is those DNA sequences having enzymatic activity that are derived from physiological conditions that are preferred for use in connection with the cassette of the present invention.

When the elements comprising the set of genetic elements of the present invention are incorporated into a vector for expression in a target cell, it is preferred that the vector contain other specialized genetic elements to facilitate the identification of cells that carry the vector and cassette and/or to increase the level of expression of the set of genetic elements comprising the cassette. The specialized genetic elements include selectable marker genes so that the vector can be transformed and amplified in a prokaryotic system. For example, the most commonly used selectable markers are genes that confer to the bacteria (e.g., *E. coli*) resistance to antibiotics such as ampicillin, chloramphenicol, kanamycin (neomycin), or tetracycline. It is also preferred that the vector contain specialized genetic elements for subsequent transfection, identification and expression in eukaryotic systems. For expression in eukaryotic cells, multiple selection strategies (e.g., Chinese Hamster Ovarian: CHO) may be used that confer to the cell resistance to an antibiotic or other drug or alter the phenotype of the cell such as morphological changes, loss of contact inhibition, or increased growth rate. Selectable markers used in eukaryotic systems include, but are not limited to, resistance markers for Zeocin, resistance to G418, resistance to aminoglycoside antibiotics, or phenotypic selection markers such *β-gal* or green fluorescence protein.

Incorporation of these components into the plasmids comprising the expression system of the present invention allows two convenient methods for removing predetermined vector sequences after the production of ssDNA. In the first method, the cassette is reverse transcribed from the PBS and the SOI between the IR comprises the loop portion of the ssDNA stem-loop intermediate that is produced when the nucleotides comprising the IR pair up to form the stem of the stem-loop vector, the stem comprising an RE site, and after digestion with the appropriate RE, the loop is released as linearized, single-stranded cDNA without (and/or with minimal) flanking sequences. In the second method, the cassette is reverse transcribed from the PBS and an SOI included in the cassette 3' to the IR is likewise transcribed, but reverse transcription is terminated at the stem of the stem-loop structure formed by pairing of the nucleotides of the IR. Either way, the resulting ssDNA is produced without (and/or with minimal) flanking sequences. If it is desired to produce ssDNA utilizing the second method, the cassette is designed with an IR that forms a stem that is more stable than the stem needs to be if the ssDNA is produced by digestion of the stem in accordance with the first aspect of the present invention (for instance, by designing the IR so as not to include an RE site). By designing the cassette with an IR that forms a stem that is easily denatured in accordance with the first aspect of the invention, reverse transcription proceeds right on through the second SOI (if it is even designed into the cassette) to the SOI located between the IR. This "premature termination" of the reverse transcriptase cDNA transcript at the 3' aspect of the stem structure therefore provides a second method for limiting the intervening vector sequences contained with an *in vivo*-produced ss-cDNA. A stem that is intermediate in stability allows production of both the first and second SOIs.

It will also be evident to those skilled in the art from this description that the intact stem-loop ss-cDNA structure can function similarly in many applications as the linearized ss-cDNA form. Consequently, the cassette is also used to advantage without the restriction endonuclease gene and associated regulatory elements and/or with a sequence of interest which lacks the corresponding restriction endonuclease site.

It will also be evident to those skilled in the art from this description of the preferred embodiments of the present invention that a cassette can be made which encodes a ss-cDNA that has a "trimmed" stem-loop structure. The RE sites encoded in the IR flanking the SOI are designed such that the stem portion (after duplex formation) is digested with the corresponding RE so as to cut the dsDNA comprising the stem in a way that removes a portion of the stem and the associated flanking sequences, yet leaves sufficient duplex DNA that the transcript retains the above-described stem-loop structure. Such a ss-cDNA structure may be more resistant to intracellular nucleases by retaining the "ends" of a ssDNA in double stranded form.

It will also be evident to those skilled in the art from this description of the preferred embodiments of the invention that the stem (duplex DNA) can be designed to contain a predetermined sequence (or sequences), i.e., aptamers, that are recognized and bound by specific DNA-binding proteins. Among other uses, such stem structure is used in the cell as a competitor to titer out a selected protein(s) that regulates specific gene function. For example, a ss-cDNA stem-loop is produced in accordance with the present invention in a cell that contains a binding site for a selected positive transcription factor such as adenovirus E1a. Adenovirus E1a, like other oncogenes, modulates expression of several adenoviral and cellular genes by affecting the activity of cell-encoded transcription factors, resulting in the changing of normal cells to transformed cells. Jones, *et al.*, 2 Genes Dev. 267-281 (1988). The duplex stem of the stem-loop intermediate produced in accordance with the present invention is therefore designed to function to "bind up" this transcription factor, preventing the protein from binding a promoter, and thus inhibiting expression of the particular deleterious gene. To those skilled in the art, it will be clear that the duplex stem structure may optionally contain multiple binding sites, for example, sites that are recognized by various transcription factors that actively regulate expression of particular gene. For example, adenovirus E1a has been found to repress transcription of the collagenase gene via the phorbol ester-responsive element, a promoter element responsible for the induction of transcription by 12-O-tetradecanolyphorbol 13-acetate (TPA), by a number of other mitogens, and by the *ras*, *mos*, *src*, and *trk* oncogenes.

The mechanism involves inhibition of the function of the transcription factor family AP-1. Offringa, *et al.*, 62 Cell 527-538 (1990). Any desired nucleotide sequence can be inserted into the genetic element that encodes the "loop" portion of the stem-loop intermediate to carry out a desired inhibitory function, e.g., antisense binding, down regulation of a gene, and so on as herein described.

In another aspect which will be recognized by those skilled in the art, the present invention is used to construct complex secondary ssDNA structures that confer biologic reactions on the cDNA transcript based on conformational secondary structure folding. Such secondary structure can be engineered to serve any of several functions. For instance, the sequence of interest may include (but is not limited to) a sequence that is incorporated into the loop portion of the single-stranded cDNA transcript to form so-called "clover leaf" or "crucible"-like structures such as those found in the long terminal repeats of adeno-associated virus or in retrotransposons. Under correct circumstances, such structure is integrated in site-specific manner into the host genome.

Because the cassette of the present invention is adaptable for incorporation into multiple commercially available delivery vectors for mammalian and human therapeutic purposes, multiple delivery routes are feasible depending upon the vector chosen for a particular target cell. For example, viral vectors are frequently used for transforming the patient's cells and introducing DNA into the genome. In an indirect method, viral vectors carrying new genetic information are used to infect target cells removed from the body and the infected cells are then re-implanted (i.e., *ex vivo*). Direct *in vivo* gene transfer into postnatal animals has been reported for formulations of DNA encapsulated in liposomes and DNA entrapped in proteoliposomes containing viral envelope receptor proteins. Nicolau, *et al.*, 80 Proc. Natl. Acad Sci USA 1068-1072 (1983); Kaneda, *et al.*, 243 Science 375-378 (1989); Mannino, *et al.*, 6 Biotechniques 682-690 (1988). Positive results have also been described with calcium phosphate co-precipitated DNA. Benvenisty and Reshef, 83 Proc. Natl. Acad. Sci. USA 9551-9555 (1986). Other systems that are used to advantage to administer the expression system including the set of genetic elements of the present invention include intravenous,

intramuscular, and subcutaneous injection, as well as direct intra-tumoral and intra-cavitary injection. The cassette, when inserted into the expression system of choice is also advantageously administered through topical, transmucosal, rectal, oral, or inhalation-type methods of delivery.

The cassette of the present invention is advantageously employed to deliver anti-sense, triplex, or any other inhibitory nucleic acid or single-stranded nucleotide sequence of interest, using known digestion and ligation techniques to splice the particular sequence of interest into the cassette (between inverted tandem repeats or between PBS and inverted tandem repeats). Those skilled in the art who have the benefit of this disclosure will also recognize that the above-described signals used for expression within eukaryotic cells may be modified in ways known in the art depending upon the particular sequence of interest. For instance, a likely modification is to change the promoter so as to confer advantageous expression characteristics on the cassette in the system in which it is desired to express the sequence of interest. There are so many possible promoters and other signals, and they are so dependent on the particular target cell for which the sequence of interest has been selected, that it is impossible to list all the potential enhancers, inducible and constitutive promoter systems, and/or poly(A) tailing systems which may be preferred for a particular target cell and sequence of interest.

In one particularly preferred embodiment, the present invention takes the form of a kit comprised of a plasmid having the above-described RNA-dependent DNA polymerase and RE genes cloned therein as well as a multiple cloning site (MCS) into which the user of the kit inserts a particular SOI. The cloning site into which the SOI is inserted is located between the above-described IR. The resulting plasmid is then purified from the cell culture in which it is maintained, lyophilized or otherwise preserved for packaging and shipping to the user. The kit preferably also includes the RE(s) for the MCS into which the SOI is to be cloned, the ligases and other enzymes, along with suitable buffers, for ligating the SOI into the plasmid, and a map of the plasmid.

In the specific embodiments described herein, the SOI(s) is/are delivered to a host cell either by co-transfection of the cells with two plasmids, designated A and B, each plasmid being designed and constructed to include the components listed above, or by a single C or D plasmid. In the two plasmid system, the B plasmid encodes the cassette including the SOI, either nested within flanking sequences that include the IR or between the IR and the PBS that provides the post-transcriptional processing signals that mediate the conversion of the mRNA into ssDNA. Activities required for processing the primary gene product of the B plasmid into ssDNA, with the removal of vector sequences and processing signals, specifically the RT/RNAse H, and RE (if utilized), are expressed from the A plasmid. The single-stranded DNA sequence that is released by interaction of the transcriptional products of these components *in vivo* is free to bind intracellular targets such as mRNA species and DNA promoters in antisense and triplex strategies.

As noted above, as described herein, the B plasmid includes cloning sites (*Not*I sites were utilized in the B plasmid described herein) between which any DNA SOI is placed (as noted above, in the examples described herein, the SOI is an antisense sequence to *c-raf* kinase including the 10-23 enzyme sequence, but as described above, other sequences that have been produced *in vivo* using the expression system described herein include a "stuffer," or test, sequence, telomeric repeats, h-ras, a region encoding the angiogenic growth factor pleiotrophin, and the region encoding tat (from SIV)). Flanking the cloning sites are signals directing the processing of the primary mRNA transcript, produced from a promoter (a CMV promoter was utilized in the B plasmid described herein), into the desired single-stranded inhibitory nucleic acid. After cloning the desired SOI into the B plasmid, the A and B plasmids are co-transfected into a cell line of choice for constitutive expression of ssDNA. Similarly, in the single plasmid expression system described herein, the SOI is cloned into that plasmid and transfected into the cell line for further processing. Regardless of the distribution of the elements of the above-described set of genetic elements between two (or even more) plasmids, or if the elements are all contained in a single plasmid, this processing

proceeds in three steps following transcription of the single-stranded DNA region (i.e., SOI, IR, and PBS):

(1) reverse transcription of the plasmid RNA transcript by RT, which in the embodiments described herein is an RT expressed by the A, C, or D plasmid (in the embodiment described herein, the RT is MoMuLV RT), proceeding from the primer binding site lying 3' to the SOI (the SOI optionally including the sequence with enzymatic activity), IR, and PBS as shown in Fig. 1;

(2) RNAse H digestion of the resulting heteroduplex, either by RNAse H activity of the RT polyprotein or by endogenous RNAse H activity, to release the single-stranded DNA precursor from its RNA complement; and

(3) Removal of flanking sequences by either digestion of the stem of a stem-loop intermediate formed upon Watson-Crick base pairing of the bases comprising the IR or by premature termination of the cDNA transcript by formation of the stem-loop secondary structure by the self-complementary IR.

Those skilled in the art will recognize from this disclosure that the particular cloning sites flanking the SOI, the particular RT, RE (if utilized), promoter, PBS, and all the other elements of the set of genetic elements of the present invention are chosen depending upon the particular SOI and/or system in which the ssDNA is to be expressed.

EXAMPLES

Except where otherwise indicated, standard techniques as described by Seabrook, *et al.* (1989) (J. Seabrook, *et al.*, Molecular Cloning: A Laboratory Manual (2nd Ed.), Cold Spring Harbor Press (1989), hereinafter referred to as "Maniatis, *et al.* (1989)") and Ausubel, *et al.* (1987) (F.M. Ausubel, *et al.*, Current Protocols in Molecular Biology, New York: John Wiley & Sons (1987)), both of which are hereby incorporated in their entirety by this specific reference thereto, were utilized in the examples set out below. It should be understood that other methods of production of ssDNA, both by natural processes and by designed artificial methods using different enzyme products or systems, may also be utilized in connection with the method of the

present invention and that the examples set out herein are set out for purposes of exemplification and are not intended to limit the scope of this disclosure or the invention described herein.

The plasmid pcDNA3.1Zeo+ was purchased from Invitrogen Corp. (Carlsbad, CA) and plasmid pBK-RSV from Statagene (La Jolla, CA). Oligodeoxynucleotides (ODN) were synthesized by Midland Certified Reagent Co. (Midland, TX). Polymerase chain reactions (PCR) were carried out using Taq DNA polymerase purchased from Boehringer Mannheim Corp. (Indianapolis, IN) in a Robo-gradient thermal cycler (Stratagene (La Jolla, CA)). Restriction endonucleases and T4 DNA ligase were obtained from Boehringer Mannheim Corp. (Indianapolis, IN). The ODNs used are listed in the attached Sequence Listing.

All ODNs were allowed to hybridize in 1 µl (5 µg/µl in water) in separate tubes which were incubated at 70°C for 5 min and allowed to hybridize for 15 min at room temperature. Standard restriction endonuclease digests were carried out (*EcoR*I used as a negative control) with 10 units of enzyme in a total reaction volume of 15 µl and appropriate reaction buffers. DNA fragments were resolved in and isolated from agarose gels. The selection of positive clones on ampicillin plates was performed after transformation into competent XL1-Blue MRF cells (Stratagene) as described by Maniatis, *et al.* (1989). After positive clones were selected, plasmid DNA was isolated using the above-described Quiagen plasmid isolation kit.

Construction of plasmids. The construction of four expression plasmids is described. The first plasmid, pssXB (Fig. 3), was derived from pcDNA3.1Zeo(+) (Invitrogen Corp.) and contains the genetic element which encodes the ss-cDNA sequence of interest used herein. pcDNA3.1Zeo(+) was digested with restriction endonucleases *Hind*III and *Not*I at positions 911 and 978, respectively. The double-stranded linker region having compatible *Hind*III and *Not*I ends which is formed by annealing the synthetic, single stranded oligodeoxynucleotides ODN-5'-N/M(link)2-H/N and ODN-3'-N/M(link)2-H/N was ligated under standard conditions into the *Hind*III/*Not*I double-digested pcDNA3.1Zeo(+) transformed into SureII cells (Stratagene, Inc.). The ODNs were allowed to hybridize in 1 µl (5 µg/µl in water) in

Ependorf tubes that were incubated at 70°C for 5 minutes and allowed to hybridize for 15 minutes at room temperature. Appropriate clones were selected and sequenced to assure proper insertion of the linker region. The resulting plasmid was termed pssXB. pssXB is shown in Fig. 4A and is the plasmid into which the sequence of interest (Fig. 4B) is cloned. For cloning sequences of interest between the inverted tandem repeats, the two *Not*I sites at positions 935 and 978, respectively (see Fig. 4A), were used. These two sites are contained within the inverted tandem repeats. For inserting sequences of interest between the inverted tandem repeats and the primer binding site, two convenient restriction endonuclease sites, *Pac*I and *Bam*HI, at positions 1004 and 1021, respectively, were used.

The second plasmid is also a component of the two plasmid vector system described herein, pssXA (Fig. 3). This "A" plasmid contains the Mo-MuLV-RT (Shinnick, T.M., *et al.*, 293 Nature 543-548 (1981)) and restriction endonuclease genes and was derived from pBK-RSV (Stratagene), also using XL-1 Blue MRF' as the host cell. A mouse cell line expressing Moloney murine leukemia virus was obtained from the American Type Culture Collection (#CRL-1858). Viral RNA was isolated from cells in accordance with the method described in Chomczymski, P. and N. Sacchi (162 Anal. Biochem. 156-159 (1987)) using Trizol reagent (GibcoBRL) and reverse transcribed using primer 3'-RT-*Hind*III (5'-CTTGTGCACAAGCTTTGCAGGTCT-3'). The transcript was then PCR amplified using the TaqPlus long polymerase system (Stratagene) for 35 cycles: 94°C 1 min, 67°C, 1 min, and 72°C, 2.5 min. Primers used for the PCR reaction were 5'-RT-*Sac*I (5'-GGGATCAGGAGCTCAGATCATGGGAC-CAATGG-3') and 3'-RT-*Hind*III, same as used for reverse transcription. These primers include compatible *Sac*I and *Hind*III sites, respectively. The 2.4kb product obtained included the sequence of the Mo-MuLV between positions 2546 and 4908. The mature viral RT peptide is encoded by the sequence between positions 2337 and 4349 (Petropoulos, C.J., Retroviral taxonomy, protein structure, sequences and genetic maps, *in* J.M. Coffin, *et al.* (Eds.), Retroviruses, New York: Cold Spring Harbor Laboratory Press, pp. 757-805 (1997)), but the peptide truncated at the amino terminus retains full activity (Tanese, N. and

S.P. Goff, 85 Proc. Natl. Acad. Sci. U.S.A. 1777-1781 (1988)). The peptide encoded by this construct includes part of the integrase gene, which follows the RT in the MoMuLV polyprotein (Petropoulos, *supra* (1997)).

The bacterium *Moraxella bovis*, which encodes the restriction endonuclease *Mbo*II (Bocklage, H., *et al.*, 19 Nucleic Acids Res. 1007-1013 (1991)), was obtained from the American Type Culture Collection (ATCC#10900). Genomic DNA was isolated from *M. bovis* using the Stratagene DNA extraction kit following the manufacturer's instructions and used as the template DNA in the PCR. Using two primers, 5'-*Mbo*II-*Hind*III (5'-CAATTAAGGAAAGCTTTGAAAAATTATGTC-3') and 3'-*Mbo*II-*Xma*I (5'-TAATGGCCCGGGCATAGTCGGGTAGGG-3'), the *Mbo*II gene was PCR amplified from genomic DNA for 30 cycles: 94°C, 30 sec., 58°C, 1 min., 72°C, 1 min. These primers were designed to include a *Hind*III and an *Xma*I site, respectively. The 1.2 kb product, copying the *M. bovis* genome between positions 888 and 2206, contains the coding region for the *Mbo*II enzyme.

The pBK-RSV vector was digested with *Xma*I and *Nhe*I. The *Nhe*I end was converted to a *Sac*I end using linker formed by two annealed oligonucleotides, 5'-Nhe-Sac-link (5'-CTAGCGGCAAGCGTAGCT-3') and 3'-Nhe-Sac-link (5'-ACGCTTGCCG-3'). The RT and *Mbo*II amplimers were ligated through the *Hind*III site and the construct was subsequently ligated between the *Sac*I and *Xma*I sites of pBK-RSV to give pBK-RSV-RT/*Mbo*II.

To insert a flexible linker between the RT and *Mbo*II domains of the polyprotein, a fragment of pBK-RSV-RT/*Mbo*II plasmid lying between the *Ase*I and *Bgl*II sites, which encodes the 5' end of the *Mbo*II gene and part of the integrase gene, was excised and replaced with an insert containing a 6-His-linker and 5'-*Mbo*II DNA fragment deleted by the double digestion. The insert was obtained by mutually-primed DNA synthesis from two templates, Rep(+) (5'-ATACTATTAATTTTGGCAAATCATAGCGGTTATGC-TGACTCAGGTGAATGCCGCGATAATTTTCAGATTGCAATCTTTCATCAATG AATTTCAGTGATGAATTGCCAAGATTGATGTTGC-3') and Rep(-) (5'-GACGAGATC-

TCCTCCAGGAATTCTCGAGAATTCGGATCCCCCGCTCCCCACCACCACCAC CACCACCCTGCCCCGCGGATGAAAAATTATGTGAGCAACATCAATCTTGGC -3'), that have complementary sequences of 17 bases at the 3'-ends. These two oligonucleotides were annealed and extended with the modified T7 DNA polymerase (USB) and the double-stranded oligonucleotide was then digested with *Ase*I and *Bgl*II and inserted into the pBK-RSV vector to give pssXA (Fig. 3).

In a first embodiment of a single plasmid expression vector system constructed in accordance with the present invention, the pc3.1DNAZeo(+)-derived "B" plasmid and the pBK-RSV-derived "A" plasmid were fused such that resulting plasmid encoded all of the elements of the set of genetic elements of the present invention, including the ss-cDNA-encoding sequence of interest, the tandem inverted repeat, the Mo-MuLV-RT gene, and the restriction endonuclease (*Mbo*II) gene. To produce the C plasmid, plasmid pssDNA-Express-A was digested with *Sac*I *Xma*I to remove the *Mbo*II gene. A linker region comprised of oligonucleotides 5'-(link)2-Hind/Xba (5'-CCGGATCTAGACCGCAAG-CTTCACCGC-3') and 3'-(link)2-Hind/Xba (5'-GGTGAAGCTTGCGGTCTAGAT-3'), which were allowed to anneal at 70°C for 15 minutes and slowly cooled to room temperature, was ligated into the plasmid after digestion under standard conditions. Positive clones were harvested and sequenced to verify linker placement and this plasmid was then digested with *Xba* and *Hind*III. The plasmid pssDNA-Express-B was then digested with *Hind*III and *Xba* and the corresponding 300 base pair DNA fragement containing the previously described inverted tandem repeats, multiple cloning site, and PBS was cloned into the digested plasmid to give pssXC (Fig. 5A). Standard ligation reactions were performed and transformed into Sure II cells (Stratagene, Inc.). Transformed positive colonies were harvested and positive clones were identified by restriction analysis.

The sequences of interest were cloned into the multiple cloning site of pssXC by using the *BamH*I and *Pac*I sites in the multiple cloning site (Fig. 5B). Four different sequences of interest were synthesized for these constructs as described above, and similar procedures were utilized for inserting each of the four sequences of interest. Each construct was prepared by allowing the paired oligonucleotides to

anneal at 70°C for 15 minutes and cooling to room temperature, followed by ligation into the plasmid under standard conditions. After transformation into SureII cells, appropriate colonies were selected with verification by sequencing for the individual inserts.

A second plasmid was constructed for use in a single-plasmid expression system, pssXD, by combining the two plasmids, pssXA and pssXB in the following manner. pssXA, which contains the Mo-MuLV reverse transcriptase (RT), was digested with *Xma*I and *Bgl*II and the resulting *Xma*I-*Bgl*II fragment was replaced with a double-stranded DNA adaptor formed by annealing two oligos, *Xma*I-*Bgl*II-Stop 1 (5'-CCGGATCTAGACCGCAAGCTTCATTTAAA-3') and *Xma*I-*Bgl*II–Stop 2 (GATCTTTAAATGAAGCTTGCGGTCTCGAT-3'). This adaptor contains a protein translation stop codon and subcloning sites, *Xba*I and *Hind*III. The resulting plasmid was designated as pssXD (Fig. 6A). *Xba*I-*Hind*III fragments were cleaved from both pssXB and pssXB-II and then cloned into pssXD between *Xba*I and *Hind*III. These DNA fragments contain: 1) RT primer binding site (PBS); 2) stem-loop structure; and 3) random control sequence (pssXB) or c-raf DNA enzyme sequence (pssXB-II). The resulting plasmids were designated as pssXD-I and pssXD-II, respectively. A RSV promoter regulates gene expression of all elements necessary for single-stranded DNA expression and all elements are transcribed as a single mRNA molecule. Endogenous $tRNA^{pro}$ binds to the PBS on the 3' end of the transcript, and is used as the primer for single-stranded DNA synthesis (Marquet, *et al.*, 77 Biochimie 113-124 (1995)). After reverse transcription of the single-stranded DNA by RT, the ssDNA is released when the template mRNA is degraded either by endogenous RNase H or the RNase H activity of the RT (Tanase and Goff, 85 Proc. Natl. Acad. Sci. U.S.A. 1777-1781 (1988)).

Tissue culture studies. Stable and transient transfections were carried out by using DOTAP liposomal transfection reagent (Boehringer Mannhiem Corp., Indianapolis, IN) using the manufacturer's accompanying instructions. All plasmid constructs were transfected into A549 lung carcinoma cell line (ATCC CCL-185) and HeLa cell lines maintained in Dulbecco's Modified Eagles Medium (DMEM)

supplemented with 10% Fetal Bovine Serum (FCS) (GibcoBRL, Gaithersburg, MD). Assays for ssDNA were performed by PCR and by dot-blot analyses 24-48 hours after transfection. ssDNA was isolated from cells transfected 48-72-hr earlier. The ss-cDNA, which co-localizes with total RNA (Mitrochnitchenko, O., *et al.*, Production of single-stranded DNA in mammalian cells by use of a bacterial retron, 269 J. Biol. Chem. 2380-2383 (1994)), was carried out using Trizol reagent (Gibco Life Technologies, Gaithersburg, MD). Assays for specific ss-cDNA species were carried out by both PCR based assays for internal fragment and by denatured single stranded gel electrophoresis with subsequent nylon blotting and probing with an internal biotin-labeled probe.

In more detail, reverse transcriptase activity was assayed using the RT-PCR assay developed by Silver, J., *et al.* (An RT-PCR assay for the enzyme activity of reverse transcriptase capable of detecting single virions, 21 Nucleic Acids Res. 3593-3594 (1993)), with modifications as set out below.. pssXA transfected cells were lysed with lysis buffer (1% Triton™, 1 mM $MgCl_2$, 100 mM NaCl, 10 mM TRIS-HCl, pH 8.0 and 2 nM DTT). After centrifugation at 18,000g for 30 min., the supernatant was collected and frozen at –80°C until use. Brome mosaic virus (BMV) RNA, used as a template, was reverse transcribed by incubation with the lysate, which would contain RT activity, for 10 or 30 min. at 37°C. Using primers 5'-CGTGGTTGACACGCAGACCTCTTAC-3' and 5'-TCAACACTGTA-CGGCACCCGCATTC-3', the product of the reverse transcription was then PCR amplified for 40 cycles: 94°C, 20 sec., 56°C, 20 sec., and 72°C, 20 sec. RT-PCR products were analysed by 1.5% agarose gel as shown in Fig. 6.

This RT-PCR assay relies upon RT activity in the cell lysates of transfected cells to produce a cDNA transcript of the BMV RNA substrate. The replication cycle of this virus does not involve a DNA intermediate, eliminating the possibility that an amplification product could be produced without prior reverse transcription. RT activity was determined in the lysates of A549 cells transfected with the pssXA plasmid (lanes 3 and 4) and the E10 clone, which showed relatively high expression (lanes 5 and 6). RT activity was also determined from A549 cells transiently transfected with

control pBK-RSV plasmid (lanes 1 and 2). For transient transfection, lysates were prepared 48 hours after transfection. Results show that cell lysates from both transient and stable transfected (E10) cells support the production of a band of expected size, 150 bp (lanes 3-6), whereas control lysates showed none (lanes 1 and 2).

To detect ssDNA expressed in mammalian cells by the pssXB-I and pssXB-II plasmids when co-transfected with pssXA into A549 cells (E10), a PCR reaction was carried out using T7 primer and c-raf DNA enzyme specific primer 5'-CTAGCTACAACGAGACATGC-3'. Total RNA fraction was used as template and pre-treated with either S1 nuclease or RNAse A for 30 min. at 37°C or left untreated. The pre-treated RNA samples were then PCR amplified for 30 cycles: 94°C, 45 sec., 55°C, 45 sec., and 72°C, 30 sec. PCR products were analyzed by 8% acylamide gel as shown in Fig. 7 (lanes 1 and 3, S1 nuclease; lanes 2, 4, and 5, RNAse). A band of the expected size was produced from both treated total RNA preparations (lanes 2 and 4) and untreated preparations (data not shown). Control preparations treated with S1 nuclease, a highly specific, ssDNA endonuclease, resulted in no amplified products (lanes 1 and 3).

The existence of c-raf DNA enzymes was further confirmed by dot-blot detection of ssDNA, using the North2South Chemiluminescent Nucleic Acid Hybridization and Detection Kit (Pierce) following the manufacturer's instructions. Two μg of total RNA, isolated from cells transfected with either pssXA/pssXB-I or pssXA/pssXB-II, or pssXA or untransfected cells, was used. The sequence of c-raf specific, biotin-labeled probe is 5'-GGCCGCACTAATGCATGTCTCGTTGTAGCTA-GCCCAGGCGGGAAGTGC-3'. As shown in Fig. 8, a biotin-labeld c-raf specific oligo probe can only detect signal in the RNA preparations isolated from E10 cells transfected with pssXB-I or pssXB-II but not untransfected E10 cells or A549 cells.

To determine whether single-stranded c-raf DNA enzyme expressed with the pssXA/pssXB vector system of the present invention in mammalian cells altered c-raf mRNA expression, northern blot analysis was performed. The E10 cell line was transiently transfected with either pssXB-I or pssXB-II. At 24 and 48 hrs, cells were

harvested for total RNA preparation. Fifteen μg of total RNA was separated on denatured agarose gel for Northern blot analysis. After overnight transfer, membrane was fixed and probed with both ^{32}P-labeled c-raf DNA fragment and glyceraldehyde-3-phosphate dehydrogenase (G3PDH), a housekeeping gene. Using random-primed labeling kit from Boehringer Mannheim, c-raf probe was prepared from an IMAGE™ cDNA clone (ID 645539, Research Genetics), which includes a coding region of c-raf kinase gene from position 571 to 2028. G3PDH was also ^{32}P-labeled and used for normalization of the RNA blot. The membrane was washed with 2xSSC, 0.1% SDS for 15 min. and 0.1xSSC for 5 min. The blot was then exposed to X-ray film or quantitated by Molecular Dynamics PhophoImager™. The quantitation result of a representative experiment by phosphor-imaging is shown in graphical form in Fig. 9. Compared to controls transfected with pssXB containing unrelated sequences, pssXB-II reduces c-raf mRNA level to 81% in 24 hrs and 66% in 48 hrs. pssXB-I had a similar effect, reducing c-raf mRNA level by 35% after 48 hrs incubation. It was also observed that there was significantly more cell death (approximately by a third) in the cells transfected with pssXA/pssXB vector expressing c-raf DNA enzyme compared to the control. Only remaining adherent cells were harvested, and not those that began to "float," so the degree of mRNA reduction may be greater than the 34-36% reduction measured.

The single plasmid vector system pssXC, was transfected into HeLa cell lines. Assays for ssDNA were performed by PCR and by dot-blot analyses 24-48 hours after transfection as described above. Reverse transcriptase activity was assayed using the RT-PCR assay developed by Silver, *et al.* (*supra* (1993)) also as described above. Individual colony isolates of stably substituted HeLa cell lines (A12 and B12) were additionally assayed for RT activity. The ss-cDNA was isolated from cells transfected 48-72-hr earlier. The ss-cDNA, which co-localizes with RNA, was carried out using Trizol reagent (Gibco Life Technologies, Gaithersburg, MD). Assays for specific ss-cDNA species were carried out by both PCR based assays for internal fragment and by denatured single stranded gel electrophoresis with subsequent nylon blotting and probing with an internal biotin-labeled probe.

This experiment showed that human tissue culture cells (HeLa cell line), transfected with plasmids designed to synthesize a processed ss-cDNA, produced ss-cDNA of the predicted size. As described in the above-incorporated application Serial No. 09/397,782, the ssDNA sequence of interest produced *in vivo* from pssXC is produced from either the position between the inverted repeats after digestion of the stem of the stem-loop intermediate or from the position between the inverted repeats and the primer binding site by premature termination of the reverse transcriptase cDNA transcript at the 3' aspect of the stem structure.

Using the total RNA fraction, the expression of intracellular single-stranded *c-raf* DNA enzyme was determined by a simple dot-blot analysis. The biotin-labeled *c-raf* specific oligonucleotide probe used was synthesized by Intergrated DNA Technologies (Coralville, IA), and was used to detect signals in the RNA samples isolated from A549 cells either transfected with control pssXD-I or pssXD-II containing the *c-raf* DNA enzyme sequence. Two μg of total RNA were pretreated with RNase A to rule out any possible non-specific hybridization to RNA, and in the presence and absence of S1 nuclease for 30 min at 37°C. Subsequently, samples were loaded onto a Hybond-N+ membrane (Amersham Pharmacia Biotec, Piscataway, NJ), and fixed by UV exposure for 3 min. Hybridization and signal detection were performed using the North2South Chemiluminescent Nucleic Acid Hybridization and Detection Kit (Pierce, Rockford, IL). Fig. 11 shows that only cells transfected with pssXD-II displayed a positive signal and that in the presence of S1 nuclease, no detectable signal was observed due to the specific degradation of ssDNA enzyme by S1 nuclease.

To determine whether single-stranded DNA enzyme expressed in A549 cells altered *c-raf* mRNA levels, quantitative RT-PCR was conducted. *c-raf* mRNA was quantitated by RT-PCR as described by Li, *et al.* (7 Gene Therapy 321-328 (2000)) with some modification. Briefly, one μg of total RNA was reverse transcribed using the Reverse Transcription System (Promega Corp., Madison, WI). A fraction of the resulting cDNA was used as a template for PCR amplification. Forty cycles of PCR were conducted (95°C, 30 sec, 50°C, 30 sec, and 72°C, 60 sec) using specific primers.

The specific primer sequences used were as follows: 1) c-raf primers: 5'-TCAGAGAAGCTCTGCTAAG-3' and 5'-CAATGCACTGGACACCTTA-3'; 2) actin primers: 5'-ACCTTCTACAATGAGCTGCG-3' and 5'-GCTTGCTGATCCACATCTGC-3'. Actin was used as housekeeping gene control. Total RNA, isolated from cells transfected with either control pssXD-I or pssXD-II containing *c-raf* DNA enzyme sequence, was reverse transcripted and PCR amplified using a pair of *c-raf* specific primers. PCR amplification of actin mRNA was used as a control to normalize loading quantity among different samples. As shown in Fig. 12, a significant reduction (approximately 70-80%) of *c-raf* mRNA was detected in the cells transfected with pssXD-II (Lane 2) compared to that of control (Lane 1).

The levels of c-raf protein in A549 cells transfected with either pssXD-I or pssXD-II were assessed by Western Blot analysis. 30 μg of cell extracts were subjected to electrophoresis on a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Proteins were electrotransferred using a Mini Trans-Blot Electrophoretic Transfer Cell according to the manufacture's instructions (BioRad Laboratories, Hercules, CA) to a Hybond ECL membrane (Amersham Pharmacia Biotec, Piscataway, NJ). The membrane was subsequently blocked in a buffer containing 25 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween–20, and 5% non-fat milk and then incubated with primary and HRP-conjugated secondary antibodies for 45 min each. The polyclonal antibodies (anti-raf1) against c-raf and monoclonal antibodies (Ab-1) against actin were purchased from Calbiochem-NovaBiochem Corp. (San Diego, CA). Monoclonal antibodies (IgG1, C-2-10) against poly-ADP ribose polymerase (PARP) were purchased either from Clontech Laboratories, Inc. (Palo Alto, CA). Proteins were visualized using SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce, Rockford, IL). As shown in Fig. 13, the level of c-raf protein in control pssXD-I transfected cells (Lane 2) was similar to that of untransfected cells (Lane 3). However, cells transfected with pssXD-II expressing *c-raf* DNA enzyme (Lane 1) had lower protein levels (approximately 20-30%) of c-raf compared to the controls.

To determine whether the expression of *c-raf* DNA enzyme could induce A549 cell apoptosis, two standard apoptosis assays, genomic DNA cleavage and PARP cleavage, were performed. Genomic DNA cleavage was determined using a LM-PCR Ladder Assay Kit (Clontech Laboratories, Inc., Palo Alto, CA) according to the manufacturer's instructions. Briefly, 0.5 μg of genomic DNA was ligated to adaptors, supplied by Clontech Laboratories, Inc. with T4 DNA ligase overnight at 15°C. A fraction of adaptor-ligated DNA was used as template in LM-PCR. Twenty-five cycles of PCR (95°C, 1 min and 72°C, 3 min) with an extension of 15 min at 72°C were conducted. Genomic DNA, isolated from cells transiently transfected with either pssXD-I (control) or pssXD-II (DNA enzyme), were ligated to specific adaptors. Subsequently, LM-PCR was carried out using a *c-raf* primer and a specific primer. As shown in Fig. 14, there was a significant increase in fragmented genomic DNA in cells transfected with pssXD-II (Lane 1) compared to cells transfected with control plasmid, pssXD-I (Lane 2), or cells left transfected (Lane 3). These results suggest that the increase in fragmented genomic DNA is a result of DNA cleavage caused by suppression of *c-raf* gene expression that was altered by the presence of the *c-raf* DNA enzyme.

Another apoptosis assay, the PARP cleavage assay, was conducted using Western Blot analysis. Compared to the controls (Lanes 2-3), cells transfected with pssXD-II (Lane 1) had decreased amounts of full–length PARP (Fig. 15), again indicating the induction of cell apoptosis by suppression of *c-raf* gene expression. Similar amounts of protein were loaded per lane as determined by the presence of actin (Lanes 1-3).

* * * * *

The experiments described above demonstrate a method of production of ssDNA *in vitro* and *in vivo* by multiple stepwise reactions using eukaryotic RT reactions and various cDNA priming reactions that successfully decreased the expression of c-raf kinase *in vivo*. Those skilled in the art will recognize that the present invention is not limited to this specific embodiment. It will be recognized, for

instance, that many nucleic acid sequences may be utilized depending upon the specific target and/or mode of inhibitory action of the SOI. Similarly, the SOI may be located in either or both of the two positions, e.g., between the IR and/or between the PBS and the 3' aspect of the IR. Likewise, the SOI may or may not include a DNA enzyme sequence depending upon the particular target and/or mode of action of the SOI and/or the DNA enzyme sequence. Those skilled in the art who have the benefit of this disclosure will recognize that any desired therapeutic effect is produced by this method by transfecting the appropriate SOI into a eukaryotic cell using the vector system of the present invention. By way of example, and not limitation, the following inhibitory nucleic acid sequences are known in the art and may be utilized as the SOI to alter gene expression in accordance with the present invention:

Sequences that act as antisense oligonucleotides to one or more RNA molecules encoding one of the several dopamine receptors for therapy of Parkinson's disease. The antisense oligonucleotides bind specifically to expression-controlling sequences of such RNA molecules, thereby selectively controlling expression of one or more dopamine receptor subtypes, and alleviating the pathological conditions related to their expression;

Sequences that inhibit expression of KSHV virion protein 26, including sequences that act as antisense and/or triplex oligonucleotides for treatement of Karposi's syndrome as described in U.S. Patent No. 5,856,903.

Oligonucleotides for control of the expression of IL-8 and/or IL-8 receptor to control growth, metastasis and/or angiogenesis in tumors as described in U.S. Patent No. 5,856,903;

Oligonucleotides having a sequence of nucleotide bases specifically hybridizable with a selected sequence of a cytomegalovirus DNA or RNA, specifically, sequences targeting cytomegalovirus DNA or RNA coding for the IE1, IE2, or DNA polymerase proteins. It is preferred that such oligonucleotides have between about 5 and about 50 nucleic acid base units as described in U.S. Patent No. 5,442,049.

Oligonucleotides specifically hybridizable with RNA or DNA deriving from a gene corresponding to one of the open reading frames UL5, UL8, UL9, UL20, UL27, UL29, UL30, UL42, UL52 and IE175 of herpes simplex virus type 1 comprising nucleotide units sufficient in identity and number to effect such specific hybridization. It is preferred that the oligonucleotides be specifically hybridizable with a translation initiation site, coding region or 5' untranslated region. The oligonucleotides are designed to be specifically hybridizable with DNA, or preferably, RNA from one of the species herpes simplex virus type 1 (HSV-1), herpes simplex virus type (HSV-2), cytomegalovirus, human herpes virus 6, Epstein Barr virus (EBV) or varicella zoster virus (VZV). Such oligonucleotides are conveniently and desirably presented as a pharmaceutical composition in a pharmaceutically acceptable carrier as described in U.S. Patent No. 5,514,577. Persons skilled in the art will recognize that the particular open reading frames described for herpes simplex virus type 1 find counterparts in the other viruses named. Thus each of herpes simplex virus type 2, cytomegalovirus, human herpes virus type 6, Epstein Barr virus and varicella zoster virus are believed to have many analogous open reading frames which code for proteins having similar functions. Accordingly, the present invention is directed to antisense oligonucleotide therapy in which the oligonucleotides are directed to any of the foregoing viruses, or indeed to any similar viruses which may become known hereafter, which have one or more of such analogous open reading frames. For convenience in connection with the present invention, all such viruses are denominated as herpes viruses.

Antisense oligonucleotides to proto-oncogenes, and in particular to the c-myb gene, for use as antineoplastic and immunosuppressive agents as described in U.S. Patent No. 5,098,890.

Antisense oligonucleotides against ICAM-1 gene expression in interleukin-1 beta-stimulated cells for use as anti-inflammatory agents with activity towards a variety of inflammatory diseases or diseases with an

inflammatory component such as asthma, rheumatoid arthritis, allograft rejections, inflammatory bowel disease, various dermatological conditions, and psoriasis. In addition, inhibitors of ICAM-1, VCAM-1, and ELAM-1 may be effective in the treatment of colds due to rhinovirus infection, AIDS, Kaposi's sarcoma and some cancers and their metastasis as described in U.S. Patent No. 5,843,738. Similarly, International Application No. PCT/US90/02357 discloses DNA sequences encoding endothelial adhesion molecules (ELAMs), including ELAM-1 and VCAM-1 and VCAM-1b. The oligonucleotides designated ISIS 1570 and ISIS 2302 are specifically contemplated as being used as the sequence of interest in the method of the present invention for decreasing the metastatic potential of target cells.

Protein-binding oligonucleotides (aptamers) that specifically bind target molecules such as proteins, and particularly thrombin, in the host cell as described in U.S. Patent No. 5,840,867. These non-oligonucleotide target molecules bind nucleic acids (Blackwell, T.K., *et al.*, 250 Science 1104-1110 (1990); Blackwell, T.K., *et al.*, 250 Science 1149-1152 (1990); Turek, C. and L. Gold, 249 Science 505-510 (1990); Joyce, G.F., 82 Gene 83-87 (1989)), specifically controlling the biological activity of the protein.

Although described with reference to the figures and specific examples set out herein, those skilled in the art will recognize that certain changes can be made to the specific elements set out herein without changing the manner in which those elements function to achieve their intended respective results. For instance, the cassette described herein is described as comprising three genetic elements, a sequence of interest, a primer binding sequence, and a tandem inverted repeat, and when transfected into a target cell with a reverse transcriptase gene under control of a suitable promoter, produces the inhibitory nucleic acid sequence described herein. However, those skilled in the art will recognize that, for instance, the mouse Moloney leukemia virus reverse transcriptase gene described for use as the reverse transcriptase gene of the cassette can be replaced with other reverse transcriptase genes (the reverse transcriptase gene from human immunodeficiency virus was one such gene which was

noted above) and that promoters other than the CMV promoter described herein may be used to advantage. As noted above, the stem-loop intermediate that is formed may or may not include a restriction endonuclease site and its susceptibility to denaturation is manipulated to advantage depending upon the particular sequence of interest that is intended to be produced from that intermediate. All such changes, and others that will be made clear to those skilled in the art by this description modifications which do not depart from the spirit of the present invention, are intended to fall within the scope of the following claims.

Table #1

Oligodeoxynucleotides (ODN's)

Name: 5'-N/M(link)2-H/N
5'AGCTTGGTCGGCGGCCTTGAAGAGCGGCCGCACTCACGATAGAGTGGGAGATGGGCGCGAGAAAGTGCGGCC
GCTCTTCAAGGCCGCCGACCTTAATTAAGTCAGCGGGGGATCCTTTTTGGGGGCTCGTCCGGGATCGGGAGACC
CCT-3'

Name: 3'- N/M(link)2-H/N
5'GGCCAGGGGTCTCCCGATCCCGGACGAGCCCCCAAAAAGGATCCCCCGCTGACTTAATTAAGGTCGGCGGCCT
TGAAGAGCGGCCGCACTTTCTCGCGCCCATCTCCCACTCTATCGTGAGTGCGGCCGCTCTTCAAGGCCGCCGACC
A-3'

Name: 5'- polyNM-gaglink-(Pleio)-DNAse-1023-B/P
5'-GAT GTA AG TCG TTG TAG CTA GCC TCC CCT G -3'

Name: 3'-polyNM-gaglink-(Pleio)-DNAse-1023-B/P
5'-GAT CCA GGG GA GGC TAG CTA CAA CGA CTT ACA TCA T -3'

Name: 5'-polyNM-gaglink-(hras)-DNAse-1023-B/P
5'-GGTGGG CGCCTCGTTGTAGCTAGCCTCGGTGTGGG-3'

Name: 3'- polyNM-gaglink-(hras)-DNAse-1023-B/P
5'-GATCCCCACACCGAGGCTAGCTACAACGAGGCGCCCACCAT-3'

Name: 5'-polyNM-gaglink-(rafK)-DNAse-1023-B/P
5'-AATGCATGTCTCGTTGTAGCTAGCCCAGGCGGGA-3

Name: 3'- polyNM-gaglink-(rafK)-DNAse-1023-B/P
5'-GATCTCCCGCCTGGGCTAGCTACAACGAGACATGCATTAT-3'

Name: 5'-polyN/M-gaglink-(tat-SIV)-DNAse-1023-B/P
5'-AGATGGAGACTCGTTGTAGCTAGCCCCCTTGAGGGCAGATTGGCGCCCGAACAGGGACTTGAAGGA-3'

Name: 3'- polyN/M-gaglink-(tat-SIV)-DNAse-1023-B/P
5'-GATCTCCTTCAAGTCCCTGTTCGGGCGCCAATCTGCCCTCAAGGGGGCTAGCTACAACGAGTCTCCATCTAT-
3'

Name: 5'-(LINK)2-Hind/Xba
5'-CCG GAT CTA GAC CGC AAG CTT CAC CGC -3'

Name: 3'-(LINK)2-Hind/Xba
5'-GGT GAA GCT TGC GGT CTA GAT -3'

ODN-PMMV(+) 129 bases (#23)	5′-CTAGGTCGGCGGCCGCGAAGATTGGTGCGCACACACACAACGCGCA CCAATCTTCGCGGCCGCCGACCCGTCAGCGGGGGTCTTTCATTTGGGGG CTCGTCCGGGATCGGGAGACCCCTGCCCAGGGCC-3′
ODN-PMMV(-) 121 bases (#24)	5′-CTGGGCAGGGGTCTCCCGATCCCGGACGAGCCCCCAAATGAAAGAC CCCCGCTGACGGGTCGGCGGCCGCGAAGATTGGTGCGCGTTGTGTGTGT GCGCACCAATCTTCGCGGCCGCCGAC-3′
ODN-Test (+) 57 bases (#38)	5′-GGCCGGAAGATTGGGGCGCCAAAGAGTAACTCTCAAAGGCACGCGC CCCAATCTTCC-3′
ODN-Test (-) 57 bases (#39)	5′-GGCCGGAAGATTGGGGCGCGTGCCTTTGAGAGTTACTCTTTGGCGC CCCAATCTTCC-3′
ODN-Telo (+) 92 bases (#40)	5′-GGCCGGAAGATTGGGGCGTTAGGGTTAGGGTTAGGGTTAGGGTTAG GGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGCGCCCCAATCTTCC-3′
ODN-Telo (-) 92 bases (#41)	5′-GGCCGGAAGATTGGGGCGCCCTAACCCTAACCCTAACCCTAACCCT AACCCTAACCCTAACCCTAACCCTAACCCTAACGCCCCAATCTTCC-3′
ODN-XB(+) 51 bases	5′-GGCCTTGAAGAGCGGCCGCACTAACACCACCACAGTGCGGCCGCTC TTCAA-3′
ODN-XB(-) 51 bases	5′-GGCCTTGAAGAGCGGCCGCACTGTGGTGGTGTTAGTGCGGCCGCTC TTCAA-3′
ODN-RT (+) 32 bases (#13)	5′-GGGATCAGGAGCTCAGATCATGGGACCAATGG-3′
ODN-RT (-) 24 bases (#12)	5′-CTTGTGCACAAGCTTTGCAGGTCT-3′
ODN-N>S (+) 18 bases (#25)	5′-CTAGCGGCAAGCGTAGCT-3′
ODN-N>S (-) 10 bases (#26)	5′-ACGCTTGCCG-3′
ODN-Mbo (+) 30 bases (#16)	5′-CAATTAAGGAAAGCTTTGAAAAATTATGTC-3′
ODN-Mbo (-) 27 bases (#33)	5′-TAATGGCCCGGGCATAGTCGGGTAGGG-3′
ODN-HisPro (+) 43 bases (#36)	5′-AGCTGGATCCCCCGCTCCCCACCACCACCACCACCCTGCCCCT-3′
ODN-HisPro (-) 42 bases (#37)	5′-AGCAGGGGCAGGGTGGTGGTGGTGGTGGGGAGCGGGGGATCC-3′
ODN-Rep(+) 121 bases	5′-ATATCTATTAATTTTGGCAAATCATAGCGGTTATGCTGACTCAGGT GAATGCCGCGATAATTTTCAGATTGCAATCTTTCATCAATGAATTTCAG TGATGAATTGCCAAGATTGATGTTGC-3′
ODN-Rep(-) 111 bases	5′-GACGAGATCTCCTCCAGGAATTCTCGAGAATTCGGATCCCCCGCTC CCCACCACCACCACCACCACCCTGCCCCGCGGATGAAAAATTATGTGAG CAACATCAATCTTGGC-3′

1. Name: 3'-RT/Mol-Hind III (24-mer)

Sequence: 5'-CTT GTG CAC AAG CTT TGC AGG TCT-3'

2. Name: 5'-RT/Mol-Sac I (32-mer)

Sequence: 5'-GGG ATC AGG AGC TCA GAT CAT GGG ACC AAT GG-3'

3. Name: 5'-Mbo II-Hind III (30-mer)

Sequence: 5'-CAA TTA AGG AAA GCT TTG AAA AAT TAT GTC-3'

4. Name: 5'-RT-Not-Mbo-Link (129-mer)

Sequence: 5'- CTA GGT CGG CGG CCG CGA AGA TTG GTG CGC ACA CAC ACA ACG CGC ACC AAT CTT CGC GGC CGC CGA CCC GTC AGC GGG GGT CTT TCA TTT GGG GGC TCG TCC GGG ATC GGG AGA CCC CTG CCC AGG GCC

5. Name: 3'-RT-Not-Mbo-Link (121-mer)

Sequence: 5'—CT GGG CAG GGG TCT CCC GAT CCC GGA CGA GCC CCC AAA TGA AAG ACC CCC GCT GAC GGG TCG GCG GCC GCG AAG ATT GGT GCG CGT TGT GTG TGT GCG CAC CAA TCT TCG CGG CCG CCG AC-3'

6. Name: 5'-Nhe-Sac-Link (18-mer)

Sequence: 5'-CTA GCG GCA AGC GTA GCT-3'

7. Name: 3'-Nhe-Sac-Link (10-mer)

Sequence: 5'-ACG CTT GCC G-3'

8. Name: 3'-Mbo II-Xba I (27-mer)

Sequence: 5'-TAA TGG CCC GGG CAT AGT CGG GTA GGG -3'

9. Name: 5'-Hind-link-Histag (43-mer)

Sequence: 5'-A GCT GGA TCC CCC GCT CCC CAC CAC CAC CAC CAC CCT GCC CCT-3'

10. Name: 3'-Hind-link-Histag (42-mer)

Sequence: 5'-AGC AGG GGC AGG GTG GTG GTG GTG GTG GGG AGC GGG GGA TCC-3'

11. Name: 5'-Not-link-test1 (57-mer)

Sequence: 5'-G GCC GGA AGA TTG GGG CGC CAA AGA GTA ACT CTC AAA GGC ACG CGC CCC AAT CTT CC-3'

12.. Name: 3'-Not-link-test1 (57-mer)

Sequence: 5'-GGC CGG AAG ATT GGG GCG CGT GCC TTT GAG AGT TAC TCT TTG GCG CCC CAA TCT TCC-3'

13. Name: 5'-Not-Mbo-link-telo (92-mer)

Sequence: 5'-GGC CGG AAG ATT GGG GCG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG CGC CCC AAT CTT CC-3'

14. Name: 3'-Not-Mbo-link-telo (92-mer)

Sequence: 5'-GGC CGG AAG ATT GGG GCG CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CGC CCC AAT CTT CC-3'

15. 5'-SL-linker-Fok1-RT (111-mer)

Sequence:5'-CTA GTC GGA TGC GGC CGC TGC ACA ACA ACA CAC AAC ACA GCG GCC GCA TCC GAT CAG CGG GGG TCT TTC ATT TGG GGG CTC GTC CGG ATC GGG AGA CCC CTG CCC AGC GCC-3'

16. 3'-SL-linker-Fok1-RT (103-mer)

Sequence:5'-CTG GGC AGG GGT CTC CCG ATC CGG ACG AGC CCC CAA ATG AAA GAC CCC CGC TGA TCG GAT GCG GCC GCT GTG TTG TTT GTT GTT GTG CAG CGG CCG CAT CCG A-3'

17. Name: XmaI-BglII-Stop 1

Sequence: 5'-CCGGATCTAGACCGCAAGCTTCATTTAAA-3'

18. Name: XmaI-BrlII-Stop 2

Sequence: 5'-GATCTTTAAATGAAGCTTGCGGTCTCGAT-3'

What is claimed is:

1. A method of altering expression of an endogenous nucleic acid target sequence in a target cell comprising the steps of:

introducing a cassette comprised of a sequence of interest flanked by inverted tandem repeats and a 3' primer binding site (PBS) into a target cell, the sequence of interest being comprised of a nucleic acid sequence designed to produce a sequence of nucleic acids that binds to an endogenous nucleic acid sequence when reverse transcribed;

reverse transcribing the mRNA transcript of the cassette from the PBS to release a single-stranded cDNA transcript in the cell; and

binding the cDNA transcript to an endogenous nucleic acid target sequence to alter expression of the target sequence.

2. The method of claim 1 additionally comprising transfecting a reverse transcriptase/RNase gene into the target cell.

3. The method of claim 1 additionally comprising linearizing the transcript of the sequence of interest.

4. The method of claim 3 wherein the transcript of the sequence of interest is linearized by including a restriction endonuclease site in the inverted tandem repeat when the cDNA transcript of the sequence of interest forms a stem-loop intermediate by Watson-Crick base pairing of the inverted tandem repeat.

5. The method of claim 2 additionally comprising inducibly promoting transcription of the reverse transcriptase gene.

6. The method of claim 5 wherein transcription of the reverse transcriptase gene is promoted with a eukaryotic promoter.

7. A cDNA transcript produced by the method of claim 1.

8. A target cell in which expression of a nucleic acid target sequence is altered in accordance with the method of claim 1.

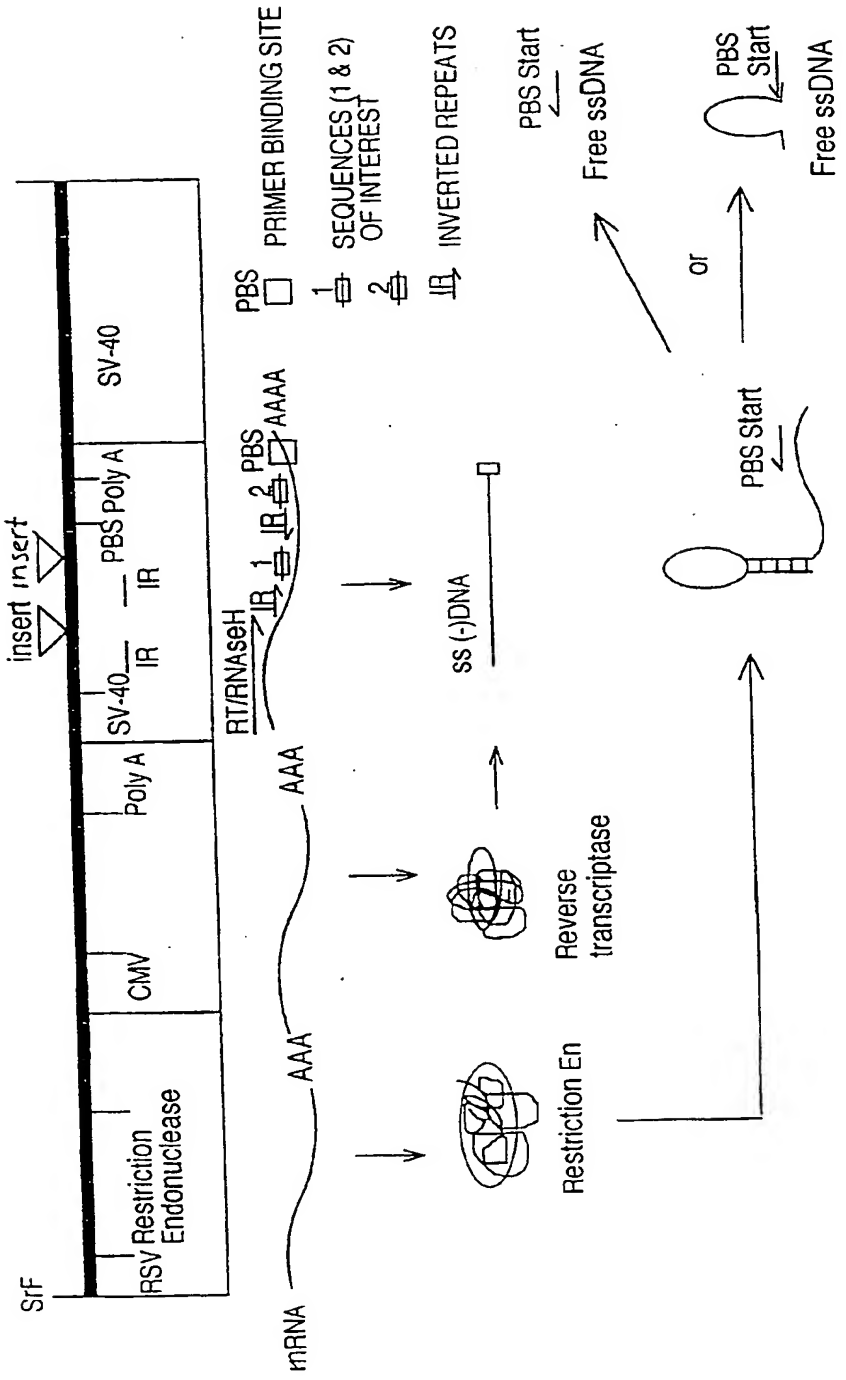
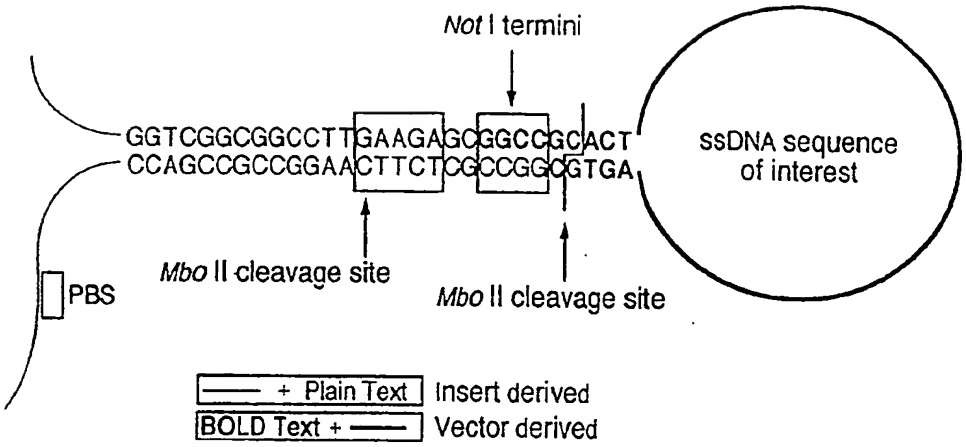

SrF
RSV
Restriction Endonuclease
CMV
Poly A
SV-40
IR
PBS
Poly A
SV-40
insert
insert
mRNA
AAA
AAA
RT/RNAseH
IR 1
IR 2
PBS
AAAA
ss (-)DNA
Reverse transcriptase
Restriction En
PBS Start
Free ssDNA
or
PBS Start
Free ssDNA
PBS
PRIMER BINDING SITE
1
2
SEQUENCES (1 & 2) OF INTEREST
IR
INVERTED REPEATS

Fig. 1



Stem-loop structure formed by single-stranded DNA

Fig. 2

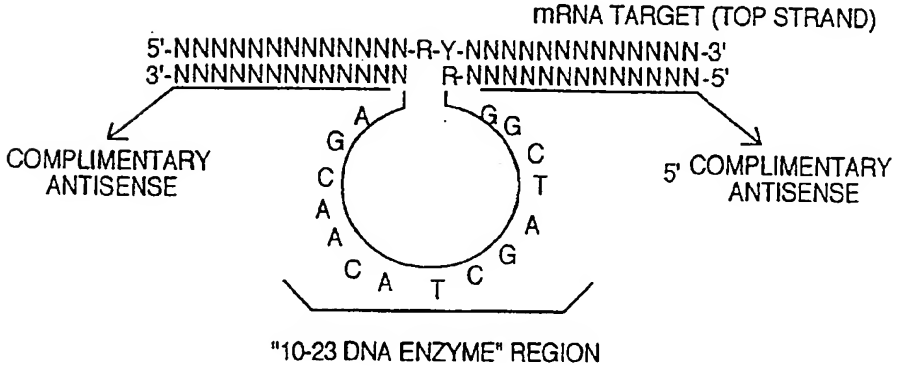


Fig. 5B

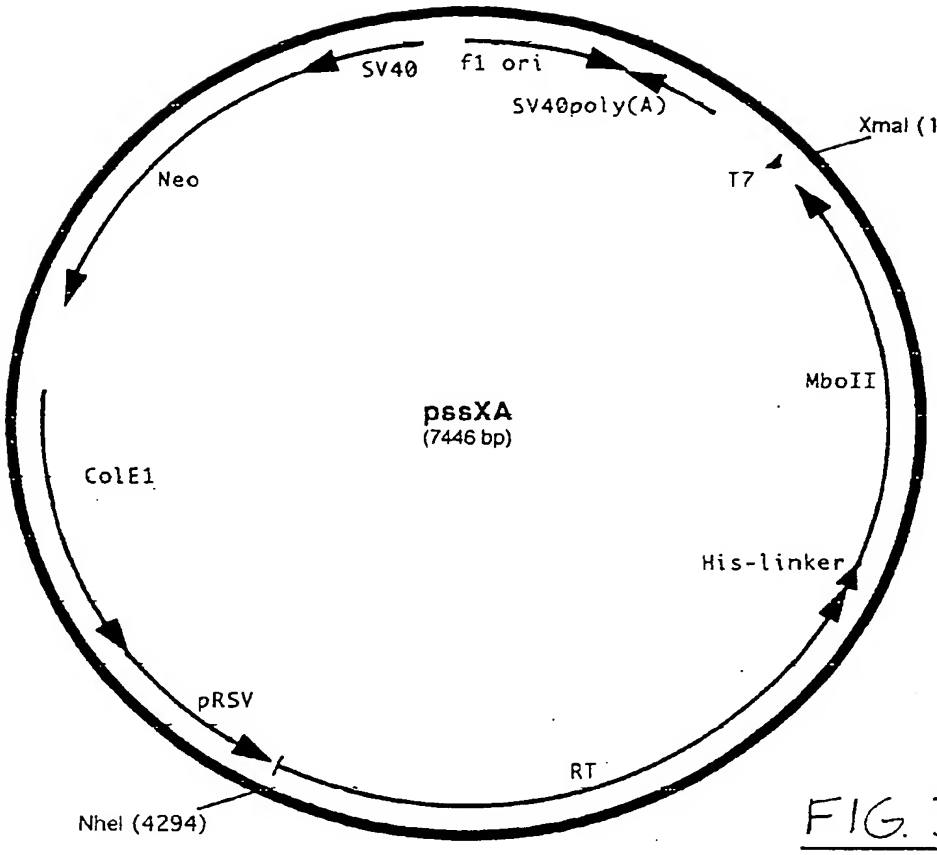

SV40
f1 ori
SV40poly(A)
XmaI (1024)
T7
Neo
MboII
pssXA
(7446 bp)
ColE1
His-linker
pRSV
RT
NheI (4294)

FIG. 3

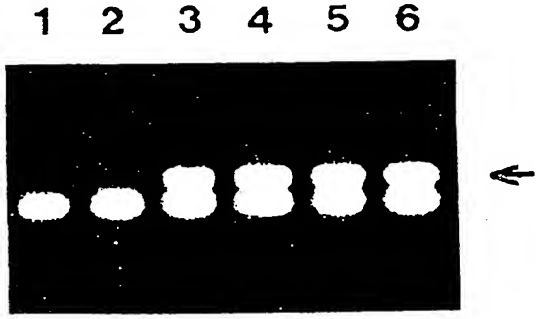

1 2 3 4 5 6

FIG. 7

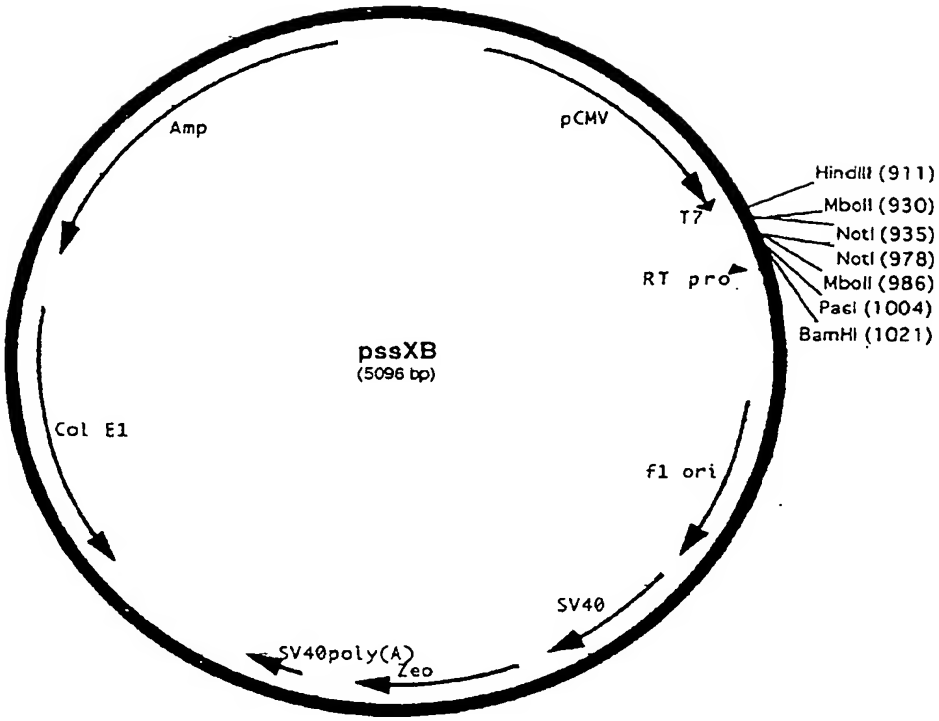
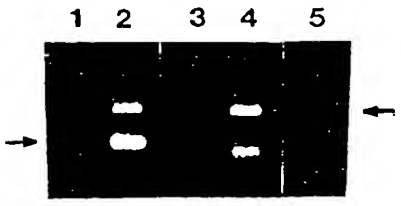

Amp
pCMV
HindIII (911)
MboII (930)
NotI (935)
NotI (978)
MboII (986)
PacI (1004)
BamHI (1021)
T7
RT pro
pssXB
(5096 bp)
Col E1
f1 ori
SV40
SV40poly(A)
Zeo

FIG. 4A

FIG. 8


1 2 3 4 5

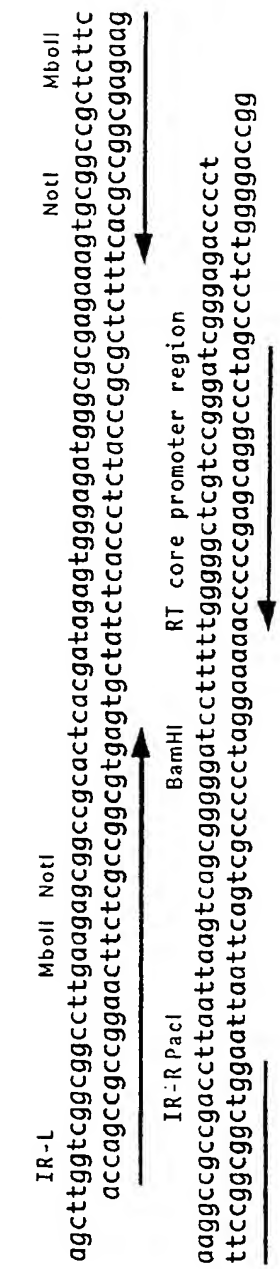

IR-L
MboII NotI
NotI MboII
1 agcttggtcggcggccttgaagagcggccgcactcacgatagagtgggagatgggcgcgagaaagtgcggccgctcttc
accagccgccggaacttctctgccggcgtgagtgctatctcaccctctacccgcgctctttcacgccggcgagaag
IR-R PacI
BamHI
RT core promoter region
10 aggccgccgaccttaattaagtcagcgggggatccttttttgggggctcgtccgggatcgggagacccct
ttccggcggctggaattaattcagtcgccccctaggaaaaaccccccgagcaggccctagccctctgggggacccgg

FIG. 4B


1
2
3
4

FIG. 9

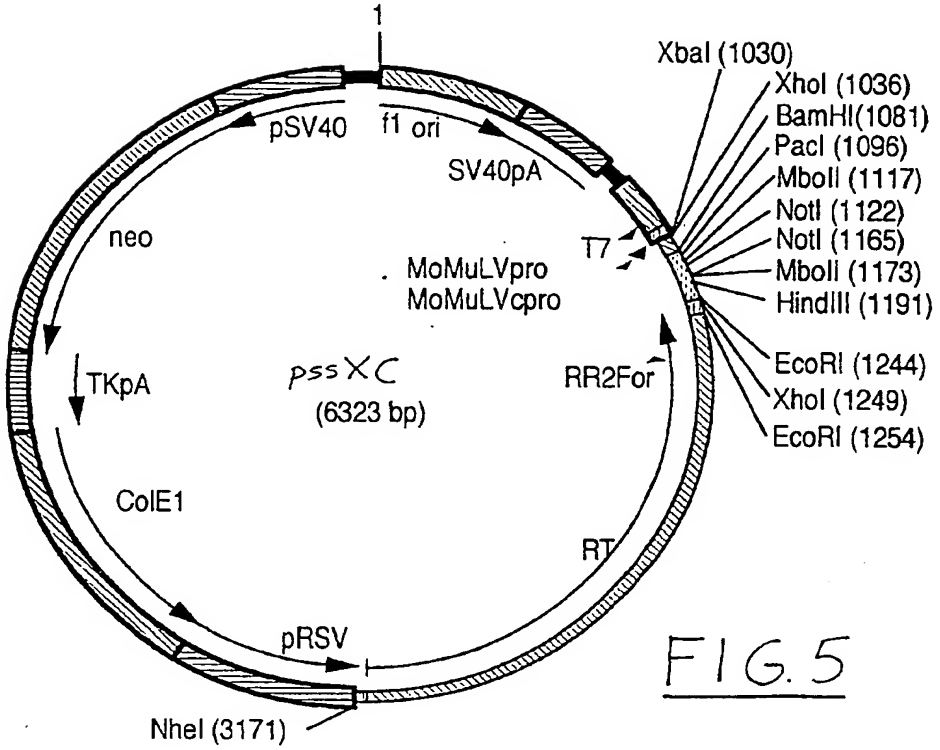

1
XbaI (1030)
XhoI (1036)
BamHI(1081)
PacI (1096)
MboII (1117)
NotI (1122)
NotI (1165)
MboII (1173)
HindIII (1191)
EcoRI (1244)
XhoI (1249)
EcoRI (1254)
pSV40
f1 ori
SV40pA
neo
T7
MoMuLVpro
MoMuLVcpro
TKpA
pssXC
(6323 bp)
RR2For
ColE1
RT
pRSV
NheI (3171)

FIG. 5

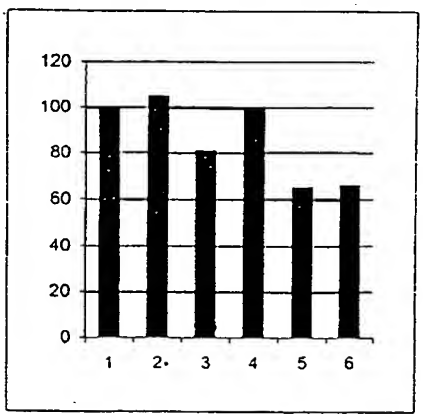

120
100
80
60
40
20
0
1
2
3
4
5
6

FIG. 10

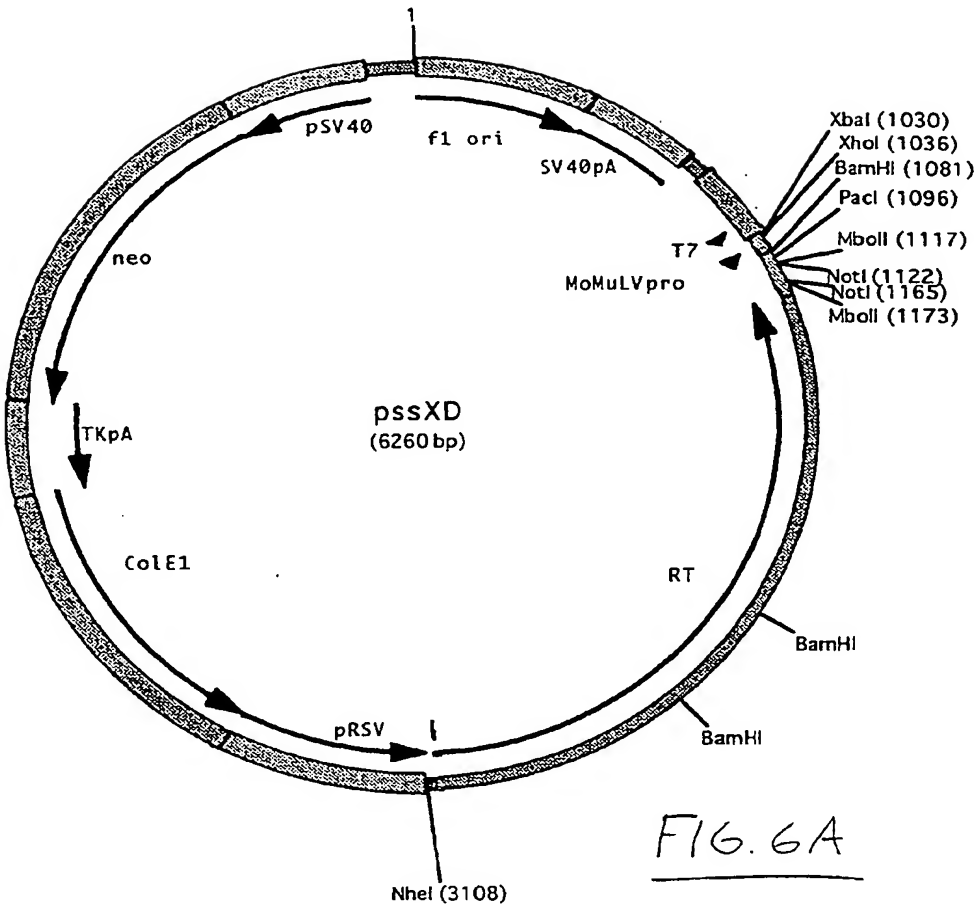
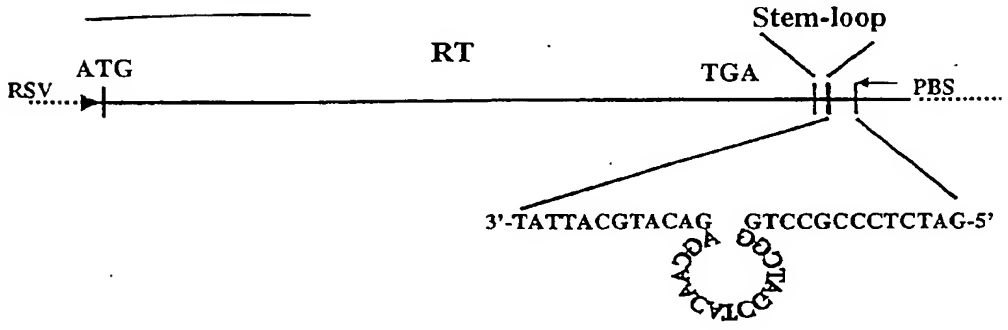

1
pSV40
f1 ori
SV40pA
neo
T7
MoMuLVpro
Xbal (1030)
Xhol (1036)
BamHI (1081)
Pacl (1096)
Mboll (1117)
NotI (1122)
NotI (1165)
Mboll (1173)
pssXD
(6260bp)
TKpA
ColE1
RT
BamHI
pRSV
BamHI
NheI (3108)

FIG. 6A

FIG. 6B


Stem-loop
RT
ATG
TGA
RSV
PBS
3'-TATTACGTACAG GTCCGCCCTCTAG-5'

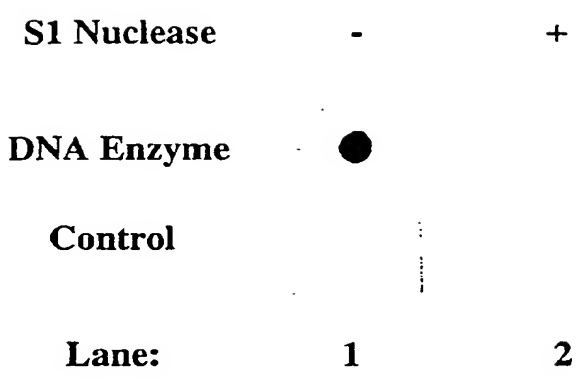


FIG. 11

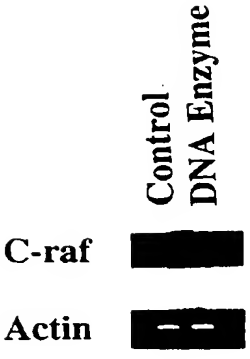


FIG. 12

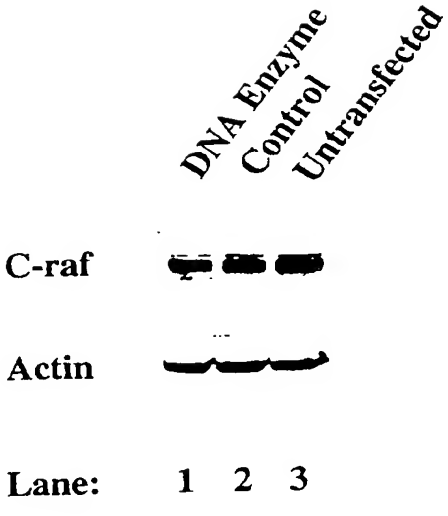


FIG. 13


DNA Enzyme
Control
Untransfected
Lane: 1 2 3

FIG. 14

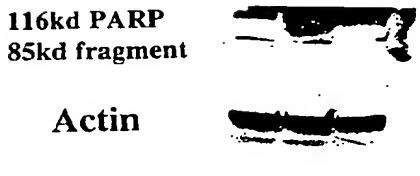

DNA Enzyme
Control
Untransfected
116kd PARP
85kd fragment
Actin
Lane: 1 2 3

FIG. 15

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/US 00/27381

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/10 C12N15/11 C12N15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, CAB Data, STRAND, BIOSIS, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X, L	WO 00 22114 A (INGENE INC ;CONRAD CHARLES A (US)) 20 April 2000 (2000-04-20) L: Priority the whole document ---	1-4
P,X, L	WO 00 22113 A (CRYOGENIC SOLUTIONS INC ;ELLISTON JONATHAN F (US); INGENE INC (US)) 20 April 2000 (2000-04-20) L: Priority the whole document --- -/--	7,8

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the international filing date

'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 March 2001	23/03/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hornig, H

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INTERNATIONAL SEARCH REPORT

Int. Jonal Application No
PCT/US 00/27381

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	CHEN YIN ET AL: "In vivo expression of single-stranded DNA in mammalian cells with DNA enzyme sequences targeted to C-raf." ANTISENSE & NUCLEIC ACID DRUG DEVELOPMENT, vol. 10, no. 6, December 2000 (2000-12), pages 415-422, XP002162673 ISSN: 1087-2906 the whole document	1-4

1

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/US 00/27381

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0022114 A	20-04-2000	AU 6298899 A	01-05-2000
		AU 6430599 A	01-05-2000
		WO 0022113 A	20-04-2000
WO 0022113 A	20-04-2000	AU 6298899 A	01-05-2000
		AU 6430599 A	01-05-2000
		WO 0022114 A	20-04-2000